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Genetic and biochemical characterization of Drosophila singed, a homolog of the actin bundling protein fascin

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Genetic and biochemical characterization of *Drosophila* singed a homolog of the actin bundling protein fascin

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ABSTRACT

Genetic and biochemical characterization of *Drosophila* singed,
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Drosophila singed mutants were named for their gnarled bristle phenotype, however, severe alleles are also female sterile due to a defect in nurse cell cytoplasm transport. Recently, singed protein was shown to have 35% peptide identity with the actin bundling protein fascin. I present evidence that singed is a homolog of fascin. *In vivo*, singed is required for actin filament bundle formation in the cytoplasm of nurse cells during oogenesis and in the cellular extension that forms a bristle. *In vitro*, purified bacterially-expressed singed bundles actin filaments with the same 12 nm periodicity and stoichiometry reported for sea urchin fascin.

While many homologs of fascin have been described, functional domain organization is not understood. I generated 15 new *sn* alleles by EMS mutagenesis; surprisingly only 2 of these expressed fascin. $singed^{G409E}$ mutants have multiply bent bristles and are fertile, although nurse cell cytoplasmic actin bundles appear disorganized and cytoplasm transport is partially incomplete. $singed^{S289N}$ mutants have gnarled bristles and are sterile. I used the $singed^{S289N}$ allele in a dominant suppressor of sterility screen and identified an intragenic mutation S251F that appears to restore much of fascin's function. These

mutations, G409E, S289N and S251F, draw attention to critical amino acids in fascin.

At wild type protein levels, both fascin and quail, a villin-like protein, are required for cytoplasmic actin bundle formation. I looked for genetic interaction between *singed* and *quail*. Mutations in *quail* enhance the intermediate egg chamber phenotype of $singed^{G409E}$. Furthermore, overexpression of quail protein can rescue the cytoplasmic actin defect and sterility of $singed^{S289N}$ and of the null allele, $singed^{28}$. In the absence of fascin, overexpression of quail is sufficient for the formation of actin bundles.

DEDICATION

This thesis is dedicated to Mrs. Ruby Littlepage with fond memories of ocean tide pools and desert reptiles. Thank you for bringing science into my childhood and continuing to support all of my endeavors.

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Chapter 1

INTRODUCTION

The cytoskeleton underlies the cell migration and alterations in cell shape that accompany morphogenesis in development and must be precisely regulated to guide these changes. Understanding cytoskeletal dynamics is central to our understanding of the exquisite mechanisms of development. *Drosophila* oogenesis and bristle development are complex events in which cytoskeletal dynamics are highly regulated and easily amenable to genetic and molecular analysis.

Oogenesis and bristle development are disrupted in *Drosophila singed* mutants. Null alleles of *singed* have gnarled bristles and are females sterile due to disruption in nurse cell cytoplasm transport. The gnarled bristle phenotype of *singed* has been an important X chromosome marker since its description by Mohr in 1922 (Mohr, 1922). The severity of the bristle defect generally correlates with the severity of the oogenesis phenotype. As described in this dissertation, these defects both result from improper organization of the actin cytoskeleton.

singed was previously cloned by Patterson and O'Hare (1991); however it did not have homology to any known protein. In 1993, the gene for the sea urchin actin bundling protein fascin was cloned and found to have 35% peptide identity with *singed* (Bryan et al., 1993). In this dissertation, I describe genetic, molecular, and biochemical investigation of *singed*. I demonstrate that singed protein is a functional homolog of sea urchin fascin and fascin is important in actin reorganization during both bristle development and oogenesis. The specific objectives of this work are the following:

1. To characterize the bristle and oogenesis phenotypes of *singed* with particular attention to the actin based cytoskeleton architecture.
2. To determine the tissue and subcellular distribution of singed protein in bristles, egg chambers, and embryos.
3. To analyze the interaction of bacterially expressed, purified singed protein with actin *in vitro*, and compare the biochemical properties of singed with those of fascin.
4. To isolate and analyze point mutations in the *singed* coding region.
5. To find dominant suppressors of the female sterility associated with *singed*.
6. To study genetic interaction between *singed* and *quail*, another mutation with effects on oogenesis similar to *singed*.

The complex process of actin reorganization that supports bristle development and nurse cell cytoplasm transport is beginning to be understood. My dissertation addresses the role of fascin *in vivo*. Many other actin binding proteins that are required for bristle development or the rapid phase of cytoplasm transport have been characterized.

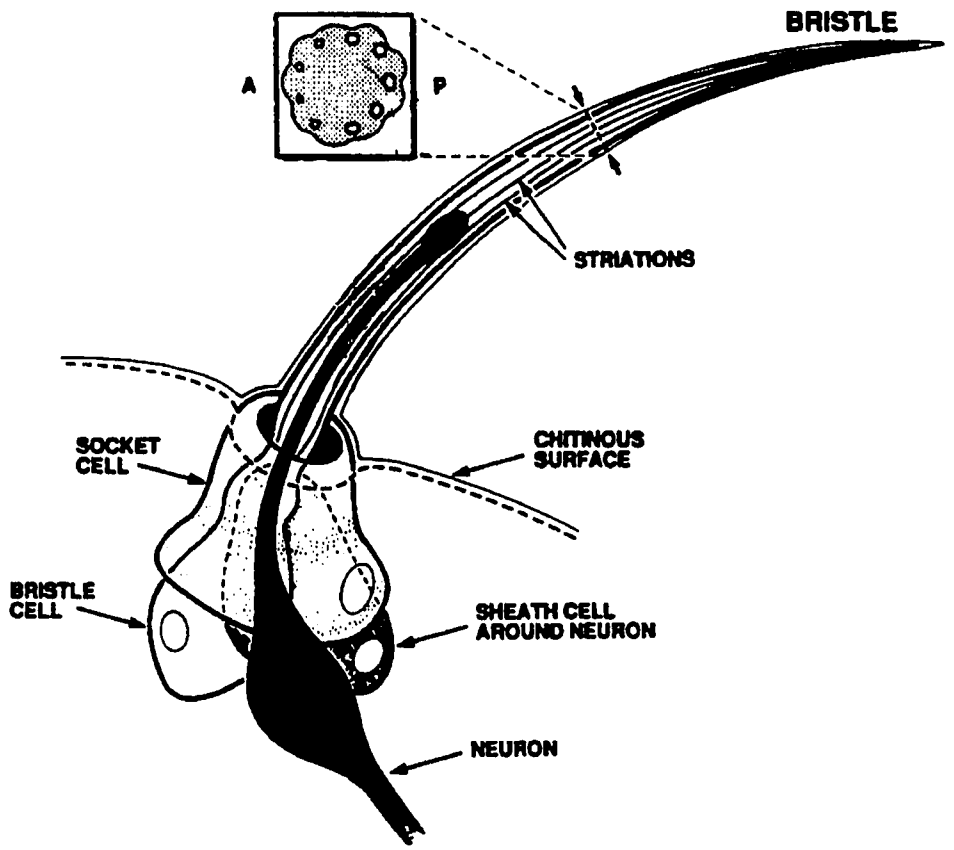
In this introduction, I will review bristle development and oogenesis as they relate to the *singed* phenotypes.

BRISTLE DEVELOPMENT

Bristles are sensory organs that develop during third instar larval and pupal stages (for review, see (Fristrom and Fristrom, 1993). Large bristles are called macrochaetae and small bristles are called microchaetae. Bristles are part of a four cell organ consisting of a bristle cell, a socket cell, a sensory neuron, and a sheath cell (glia cell) (Figure 1). These four cells arise from a mother cell that undergoes two cell divisions [Lees and Waddington, 1942]. One cell division occurs at puparium formation and the second occurs

Figure 1. Description of bristle development

Bristle develop during pupation as part of a 4 cell sensory organ that contains a bristle cell (trichogen), a socket cell (tormogen), a sensory neuron, and a sheath cell (glia cell) (drawing from Tilney et. al., 1995). The trichogen cell forms the bristle by sending out a shaft of cytoplasm that is supported by a cytoskeletal core. 8-10 actin filament bundles are located peripherally at regular intervals adjacent to the membrane (cross-sectional view, (Appel et al., 1993; Overton, 1967). Peripheral actin bundles are more dense on the posterior surface of the bristle compared to the anterior (A) surface.



between 9-18 hours after puparium formation (Hartenstein and Posakony, 1989). The cells that form the bristle and socket cell are called the trichogen and tormogen, respectively (Figure 1). Once the trichogen and the tormogen are established, they grow considerably and become polyploid by about 40 hours after puparium (Lees and Picken, 1945; Lees and Waddington, 1942).

Bristles extrusion occurs between 30 and 45 hours after puparium formation. The trichogen cell sends out a shaft of cytoplasm that is supported by a cytoskeletal core (Figure 1). Growth of a bristle appears to occur by elongation at the tips. *forked* mutants have branched bristles and the region distal to a fork continues to elongate while the region proximal to a fork is unchanged (Lees and Picken, 1945). Bundles of longitudinally oriented microfilaments are found peripherally at regular intervals adjacent to the membrane (Overton, 1967). Rhodamine-phalloidin staining of developing bristles showed that these microfilament bundles are composed of actin (Appel et al., 1993). The central core of the bristle shaft is packed with longitudinally oriented microtubules. These microtubules might function as tracks upon which proteins and vesicles can be transported to the tip of the bristle. Once bristle extension is complete, the cuticle is deposited. By 60 hours after puparium formation, the microfilament bundles disappear.

The morphology of the bristle appears to reflect the organization and integrity of the cytoskeletal core present at the time of cuticle deposition during bristle development. Aberrant bristle morphology in *singed (sn)*, *Stubble/stubbloid (Sb/sb)*, *forked (f)*, and *chickadee (chic)* mutants has been correlated with defects in the organization and composition of actin filament bundles (Appel et al., 1993; Cant et al., 1994; Overton, 1967; Petersen et al., 1994; Verheyen and Cooley, 1994, Tilney et al., 1995). In order to understand the exquisite morphological changes that allow bristle development, one

must understand the proteins that regulate the formation of the cytoskeletal core. The genes disrupted in *singed, Stubble, chickadee,* and *forked* mutants have all been cloned and their role in bristle development analyzed (Table 1).

singed (sn). *singed* bristles are gnarled. Electron microscopic cross sectional analysis of the bristle extension in mutants revealed that the microfilament bundles are very small, flat and ribbon-like rather than round and columnar (Figure 2; (Overton, 1967). The actin bundles are not hexagonally packed (Tilney et al., 1995). These small actin bundles probably cannot provide the structural support to direct bristle extension in a single direction. As discussed in this dissertation, *singed* encodes *Drosophila* fascin, an actin bundling protein, and is required for actin filament bundle formation in bristles (Bryan et al., 1993; Cant et al., 1994).

Stubble/stubbloid (Sb/sb). *Stubble* is a dominant mutation and *sb* is a recessive mutation in the same gene. Bristles on *Stubble* mutants are less than one-half the length of wild type bristles, thick with blunted ends. Bristles on *stubbloid* mutants have frayed ends and are short, although a little longer than *Stubble* bristles. Rhodamine-phalloidin staining of developing mutant bristles showed that *stubbloid* bristles contained actin bundles that become disorganized at the tips. Both rhodamine-phalloidin staining and EM cross sectional analysis of extending *Stubble* bristles demonstrated that the actin bundles are smaller and more numerous (Figure 2; (Overton, 1967, Appel, 1993 #427). *Stubble* encodes a transmembrane protein with an extracellular serine protease domain (Appel et al., 1993). Unfortunately, antibodies to Stubble/stubbloid are not available and the protein localization is unknown. The extracellular protease domain may allow the stubble protein to degrade the extracellular matrix and support cell

detachment. The intracellular domain of this protein may modify the cytoskeleton in response to extracellular cues.

forked (f). *forked* bristles twist, bend and split. They are phenotypically similar to *singed* bristles. Electron microscopic cross-sectional analysis of the bristle extension in *forked* mutants revealed that the actin microfilament bundles were extremely small, but unlike in *singed*, retained hexagonal packing (Figure 2; (Tilney et al., 1995). The *forked* gene encodes 6 transcripts (6.4, 5.6, 5.4, 2.5, 1.9, and 1.1 kb) that are expressed during mid-late pupal development (Hoover et al., 1993). The proteins encoded by the 5.6 and 5.5 kb transcripts contain ankyrin repeats at the amino terminus and may be involved in protein-protein interactions (Hoover et al., 1993). However, sequences from this region are not required for forked gene function since transgenes lacking the ankyrin repeats can rescue forked mutants (Petersen et al., 1994). Interestingly, overexpression in wild type flies of small forked proteins encoded by the 2.5, 1.9, and 1.1 kb transcripts results in abnormal bristle extension. In wild type bristles, forked protein localizes to the actin bundles in the cytoskeleton core of the bristle extension before singed is detected (Petersen et al., 1994). Forked appears to be a structural component of the microfilament actin bundles but the predicted forked proteins do not have homology with any other known protein.

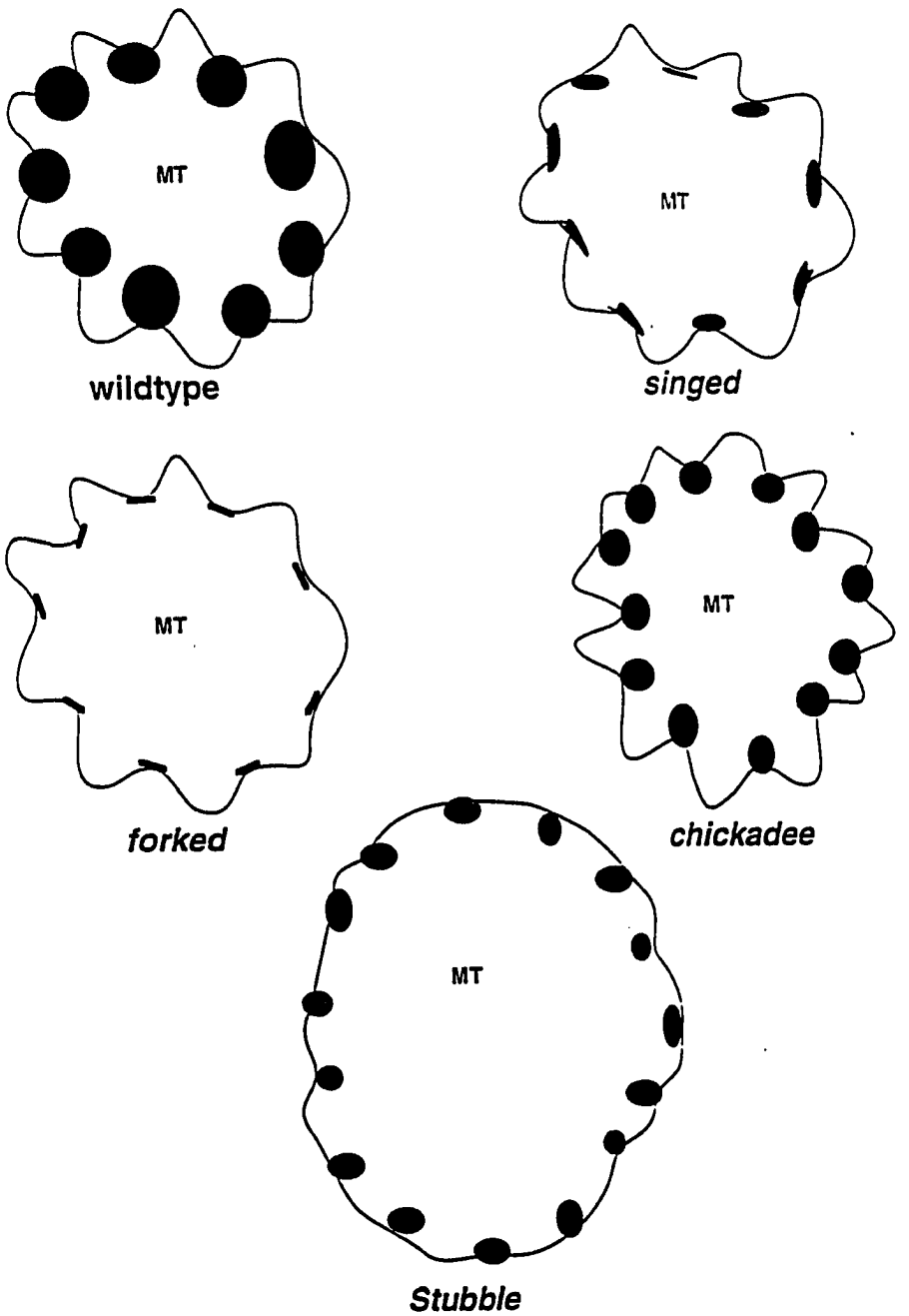
chickadee (chic). In severe *chickadee* mutants, the bristles are short and thick and the ends of the bristles are often severely bent or forked (Verheyen and Cooley, 1994). Rhodamine-phalloidin staining of the developing bristles demonstrated that more actin bundles were present and these bundles appeared thin and disorganized (Figure 2). *chickadee* encodes *Drosophila* profilin, a small actin monomer binding protein that regulates actin polymerization. Profilin is found in the cytoplasm of the bristle extension. In

Table 1: Mutations affecting bristle development

Gene	Protein	Morphology	Actin Bundles		Protein Localization
			RhPh	EM	
wild type		straight, tapered	dense, parallel actin bundles	8-12 round bundles	
singed	fascin	twisted, bent, split	faint, disorganized	8-12 thin small bundles	cytoplasm
Stubble	extracellular serine preotease domain, intracellular domain has no homology	short, blunt-ends	increased number of smaller actin bundles	increased number of smaller bundles	?
forked	no homology	split, twisted, bent	?	very small	colocalize with actin bundles
chickadee	profilin	short, split-ends	increased number of thin disorganized bundles	?	cytoplasm

Figure 2. Organization of actin bundles in bristle mutants

EM cross-sectional analysis and/or rhodamine-phalloidin staining of developing bristles revealed that actin bundles are defective in many bristle morphology mutants. The most severe defect is seen in *forked* bristles where the actin bundles are extremely small (Tilney et al., 1995). In *singed* bristles the actin bundles are very small and thin and lack the hexagonal packing seen in wild type bristles (Overton, 1967, Tilney, 1995 #819). *Stubble* bristles have a large cross-sectional area and there are more actin bundle. The bundles are smaller than wild type but maintain their columnar shape (Appel et al., 1993). Rhodamine-phalloidin staining of chickadee bristles suggests that they have an increased number of thin actin bundles (Verheyen and Cooley, 1994).



bristles, profilin appears to have a role in limiting the number of actin bundles. Based on analogy with the role of profilin in the acrosomal reaction of echinoderm sperm (Tilney et al., 1983), it was suggested that profilin may function to sequester actin monomers to control the nucleation of new actin filaments (Verheyen and Cooley, 1994).

OOGENESIS

In *Drosophila*, each ovary consists of about 16 parallel ovarioles that contain bundles of developmentally ordered egg chambers (King, 1970; Mahowald and Kambysellis, 1980) and reviewed in (Spradling, 1993). Oogenesis is initiated by a germline stem cell that resides at the anterior end of each ovariole in the germarium (Figure 3A). Each germline stem cell divides into a daughter stem cell and a cystoblast. The cystoblast subsequently undergoes four consecutive mitotic divisions to yield a 16 cell cluster. Since cytokinesis in the germline divisions is incomplete, the sixteen cells remain interconnected by a series of cytoplasmic bridges called ring canals. One of the 16 cells differentiates into the oocyte while the remaining 15 cells differentiate into highly polyploid nurse cells. Many of the proteins and transcripts that are required for oogenesis and early embryogenesis are produced in the nurse cells and then transported into the oocyte through the ring canals throughout oogenesis. In the germarium, the 16 cell germline syncytium is surrounded by somatic follicle cells to form a stage 1 egg chamber. Egg chambers leave the germarium and enter the vitellarium where they mature through morphologically distinct stages 2 through 14 (King, 1970; Mahowald and Kambysellis, 1980) and move posteriorly within the ovariole (Figure 3A).

Egg chambers increase in size during stage 2-7 of oogenesis. As nurse cell cytoplasm is slowly transported into the oocyte, the oocyte grows (Figure

3A). During stages 7 through 10, yolk proteins synthesized by the fat bodies and the follicle cells are absorbed into the oocyte by endocytosis. By stage 10 the oocyte occupies half the volume of the egg chamber (Figure 3A, B).

As the egg chamber grows, the somatic follicle cells divide to maintain a monolayer of about 1000 cells that surrounds the egg chamber. By stage 9, distinct subpopulations of follicle cells can be recognized (Figure 3B). Most of the follicle cells move posteriorly and change their shape to form a columnar epithelium near the oocyte. A thin layer of cells remains anteriorly and covers the nurse cells. A group of 6-10 cells, called border cells, at the anterior end of the egg chamber lag behind when the follicle cells begin to migrate posteriorly toward the oocyte. Border cells remain associated with each other and migrate between the nurse cells toward the anterior tip of the oocyte (Figure 3B). A small group of cells located at the posterior end of the egg chamber appear to have the same developmental potential as border cells.

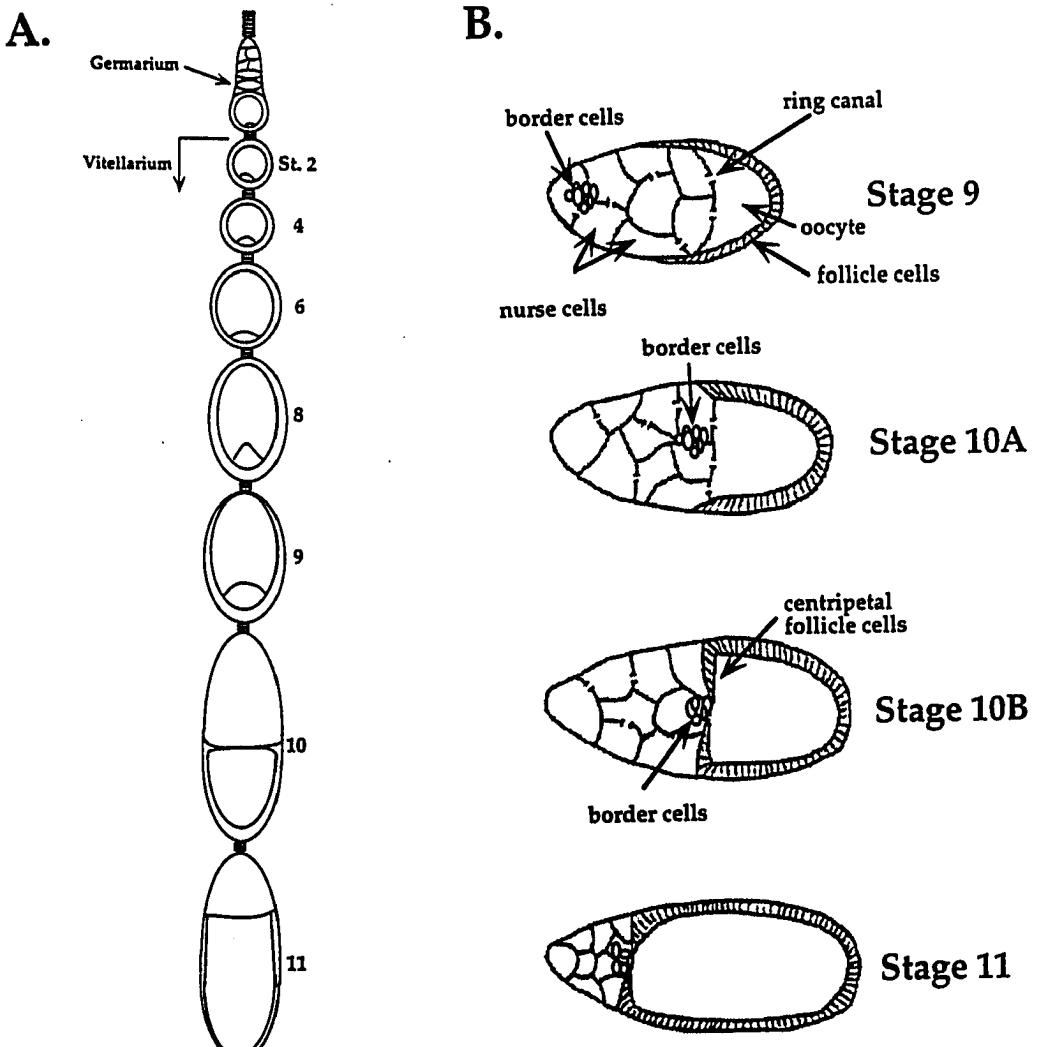
At stage 10A, the follicle cells have completed their posterior migration and the border cells have reached the nurse cell-oocyte junction. During stage 10A, follicle cells at the anterior margin of the oocyte migrate centripetally along the nurse cell-oocyte interface. Stage 10B is marked by the completion of centripetal follicle cell migration. The border cells and some of the centripetal follicle cells meet at the anterior border of the oocyte where they form the micropyle. The migration of these subsets of follicle cells provides some of the morphological criteria for identifying the correct developmental stage of an egg chamber (Figure 3B).

Stage 10 marks a critical turning point in nurse cell cytoplasm transport (for review see (Mahajan-Miklos and Cooley, 1994a). Prior to stage 10, nurse cell cytoplasm is transported slowly into the oocyte. In stage 10B, the nurse cell nuclei become permeable and a rapid phase of cytoplasm transport begins.

Figure 3. Description of oogenesis

(A) Ovarioles contain egg chambers that are maturing through 14 morphologically distinct stages (A). The oocyte grows as egg chambers mature; nurse cell cytoplasm is transported into the oocyte and the oocyte endocytoses yolk protein.

(B) Stages 9-11 are distinguished by the position of migratory follicle cells. At stage 9, most of the follicle cells begin to move posteriorly and 6-10 border cells begin to migrate between the nurse cells toward the anterior tip of the oocyte. At stage 10A, the follicle cells have completed their posterior migration and they change shape to form a columnar epithelium around the oocyte. A group of follicle cells at the anterior of the oocyte begin to migrate centripetally. At this point the oocyte is about half the volume of the egg chamber. Stage 10B, is marked by the completion of centripetal follicle cell migration along the anterior border of the oocyte. Rapid cytoplasm transport begins at stage 10B and during stage 11, all of the remaining nurse cell cytoplasm is rapid transported into the oocyte.



During stage 11, the remaining nurse cell cytoplasm, including karyoplasm, is rapidly transported into the oocyte. The oocyte doubles in size and the nurse cells fully regress in only about 30 minutes. Treatment of egg chambers with cytochalasin B prevents this rapid phase of cytoplasm transport and suggests that this rapid phase of transport is an actin dependent process (Gutzeit and Huebner, 1986).

Throughout oogenesis, actin is found in a subcortical network and in ring canals. Just prior to rapid cytoplasm transport, an additional actin network forms in the nurse cell cytoplasm. Cytoplasmic actin filament bundles form rapidly and radiate from the plasma membrane to the nuclear membrane. The cloning and characterization of *chickadee, quail, singed, spaghetti squash,* and *armadillo* has confirmed the importance of actin-based cytoskeleton in rapid cytoplasm transport. Female sterile mutations causing defects in the rapid phase of nurse cell cytoplasm transport can be classified by their cytoskeletal defect (Table 2).

Formation of nurse cell cytoplasmic actin bundles:

Mutant egg chambers from *chickadee, quail,* and *singed* (described in Chapter 3) females are phenotypically identical (Cant et al., 1994; Cooley et al., 1992b; Mahajan-Miklos and Cooley, 1994b). In these mutants, the subcortical actin and the ring canals are normal but the cytoplasmic actin filament bundles are not present (Table 2). Rapid nurse cell cytoplasm transport is initiated, but the nurse cell nuclei appear to move with the flow of cytoplasm, lodge in ring canals, and block the flow of cytoplasm into the oocyte (Cooley et al., 1992b). Eggs from these mutants remain very small and fail to develop. This phenotype suggests that the cytoplasmic actin filament bundle network anchors the nurse cell nuclei in a central position away from ring canals while the subcortical actin filament network supports myosin based nurse cell

Table 2: Mutations affecting nurse cell rapid cytoplasm transport

Gene	Protein	Egg Chamber Morphology	Actin Bundles		Nurse Cell Protein Localization
			subcortical	cytoplasmic	
wild type	NA	complete cytoplasm transport	present	dense organized	NA
singed	fascin	dumpless	present	absent	cytoplasm
quail	villin-like	dumpless	present	absent	cytoplasmic actin bundles
chickadee	profilin	dumpless	present	absent	cytoplasm
armadillo	β-catenin plaktoglobin	dumpless displaced oocyte cell shape changes	disrupted	absent	cell-cell adhesion sites
spagetti squash	regulatory myosin light chain	dumpless	present	dense organized	unknown

contraction and generates the force that drives rapid cytoplasm transport (Cooley et al., 1992b; Wheatley et al., 1995).

chickadee (chic). *chickadee* encodes *Drosophila* profilin, a small actin monomer binding protein. Extensive biochemical analysis of profilin, *in vitro*, suggest that it is involved in regulating actin polymerization and may also be involved in signal transduction (Verheyen and Cooley, 1994). The absence of nurse cell cytoplasmic actin bundles in *chickadee* females suggests that profilin may promote actin filament assembly in nurse cells (Verheyen and Cooley, 1994). Two models, which incorporate the *in vitro* properties profilin, have been proposed for the regulation of actin filament assembly in nurse cells (Verheyen and Cooley, 1994). Profilin may promote actin filament polymerization by maintaining the pool of cytoplasmic actin monomers in an ATP-bound state by stimulating nucleotide exchange on actin monomers (Goldschmidt-Clermont et al., 1992). This might ensure a maximum rate of polymerization in nurse cells because *in vitro* studies suggest that ATP-actin monomers add to growing filaments at a faster rate than ADP-actin monomers and ATP-actin monomers have a lower critical concentration (Pollard, 1986). Alternatively, profilin may be part of a signal transduction pathway that triggers the rapid polymerization of actin in stage 10B nurse cells. *In vitro*, profilin can bind phosphatidylinositol 4,5-biphosphate (PIP_2) and can inhibit PIP_2 hydrolysis by unphosphorylated phospholipase C-1(Goldschmidt-Clermont et al., 1990; Lassing and Lindberg, 1985). Phosphorylated phospholipase C- 1 can hydrolyze PIP_2 bound to profilin and presumably release profilin into cytoplasm (Goldschmidt-Clermont and Jamney, 1991b). However, profilin resides in the nurse cell cytoplasm and does not appear to accumulate in the membrane where one would expect it to colocalize with PIP_2 (Verheyen and Cooley, 1994).

quail (qua). Quail co-localizes with the subcortical actin filaments prior to the formation of the cytoplasmic actin network and then co-localizes with the cytoplasmic actin bundle networks during rapid cytoplasm transport (Mahajan-Miklos and Cooley, 1994b). *quail* shares sequence homology with the actin cross-linking protein, villin (Mahajan-Miklos and Cooley, 1994b). Villin has been shown to be a phosphoinositide and calcium regulated actin binding protein *in vitro* (reviewed in (Mooseker, 1985). Villin can cross-link actin filaments under physiological calcium concentrations (10^{-7} μM), whereas it can cap, sever, and nucleate actin filaments under high calcium concentrations (10^{-6} μM). In vertebrates, villin co-localizes with actin filament bundles in microvillar projections that constitute the brush-border on the absorptive intestinal epithelium. *Quail* sequence similarity to villin, its protein localization to actin bundles, and its requirement for cytoplasmic actin bundle assembly suggest that quail cross-links actin filaments in the nurse cell cytoplasm (Mahajan-Miklos and Cooley, 1994b). Quail protein might also support cytoplasmic actin bundle formation by promoting the nucleation of new actin filaments in the cytoplasm by capping and severing subcortical actin filaments (Mahajan-Miklos and Cooley, 1994b).

singed (sn). *singed* encodes a protein with homology to fascin, another actin binding protein. Fascin is known to bundle actin filaments in a variety of different structures. As discussed in Chapter 3, singed is expressed in the nurse cell cytoplasm where it is required for cytoplasmic actin bundle formation.

Organization of the actin based cytoskeleton in nurse cells:

armadillo (arm). The armadillo gene encodes a protein with homology to vertebrate plaktoglobin and β-catenin, two components of the vertebrate adherens junction (Franck et al., 1990; McCrea et al., 1991; Peifer and

Weischaus, 1990). Adherens junctions mediate cell-cell interactions and are thought to anchor the cytoskeleton to sites of cell-cell adhesion. Armadillo protein localized to regions of cell-cell adhesion (Peifer et al., 1993). Germline clones of *armadillo* null alleles were analyzed to determine the requirement of *armadillo* in egg chambers (Peifer et al., 1993). Nurse cells in the germline clones had severe defects in actin organization. The subcortical actin network, ring canals, and cytoplasmic actin filament network were all disrupted. These mutant egg chambers also displayed abnormalities in cell shape, displaced or irregular oocyte positioning, and failure to undergo final cytoplasm transport. These disruptions in egg chamber morphology are consistent with a role for armadillo in cell adhesion and demonstrate the importance of an intact actin cytoskeleton (Peifer et al., 1993).

Force generating mechanism:

spaghetti squash (sqh). spaghetti squash encodes the regulatory light chain of nonmuscle myosin II and is an essential gene (Karess et al., 1991). Although rapid nurse cell cytoplasm transport is disrupted in germline clones of the hypomorphic *spaghetti squash* allele, sqh^1, the actin cytoskeleton is not disrupted (Wheatley et al., 1995). The subcortical actin, ring canals, and the cytoplasmic actin bundle network are all present and the nurse cell nuclei maintain their central position in the cell. However, in these mutant clones, distribution of myosin II is abnormal (Wheatley et al., 1995). In stage 10 egg chambers from wild type females, myosin II is localized to the subcortical cytoplasm where it coincides with the subcortical actin network. In sqh^1 germline clones, myosin II is not localized to the subcortical region but rather appears to exist in particles that likely represent non-functional aggregates of myosin II resulting from the reduction in myosin regulatory light chain. The phenotype in sqh^1 clones suggests that myosin II is required to generate the

force required for rapid cytoplasm transport by mediating the contraction of nurse cells (Wheatley et al., 1995).

SUMMARY

The reduction in actin filament bundles in *singed* bristles, the failure of nurse cell cytoplasm transport in egg chambers of sterile *singed* females, and the recently described homology between singed and fascin all suggest that the singed protein may interact with the actin-based cytoskeleton. The work presented in the following chapters indicate that singed protein is a functional homolog of fascin. In addition, I identify three critical amino acids in fascin by mutational analysis. Finally, by studying the interaction between *singed* and *quail in vivo*, I are beginning to understand how two actin cross-linking proteins work together to promote the assembly and stability of nurse cell cytoplasmic actin bundles.

Chapter 2

MATERIALS AND METHODS

Drosophila stocks

All fly stocks were maintained under standard culturing conditions. *singed* alleles used in this dissertation are described in Table 3. Four spontaneous *singed* (*sn*) alleles were studied: sn^2, sn^3, sn^4, and sn^{36a}. sn^{X2}, a putative null allele, was induced by X-ray mutagenesis. Some of the sites of mutation for these alleles have been described (Paterson and O'Hare, 1991; Roiha et al., 1988). Transcription of *singed* in sn^3 uses a cryptic promoter and starts 200 bp closer to the *olfE* promoter; the developmental profile of the cryptic promoter allows adequate expression during oogenesis but not bristle formation (Paterson and O'Hare, 1991). sn^{G409E}, sn^{S289N}, and sn^{S251F} were generated by EMS mutagenesis as part of this dissertation and will be thoroughly described in Chapter 5.

For mutagenesis, males with an isogenic w^{1118} chromosome were maintained as a stock with *C(1) y f/Y* females. *FM7c, y sc w v B* sn^{X2} stock was used as null *sn* allele (Cant et al., 1994; Paterson and O'Hare, 1991; Roiha et al., 1988). $forked^{36a}$ is a severe allele that was obtained from the Indiana University stock center (Hoover et al., 1993; Petersen et al., 1994). $furrowed^1$ was obtained from the Mid-America *Drosophila* Stock Center. $cn;ry^{506}$ and Canton S flies were used as wild type controls.

qua^{HM14} and qua^{WP16} are intermediate *quail* alleles that were induced by EMS mutagenesis (Schüpbach and Wieschaus, 1991). These *quail* alleles were shown to express *quail* transcript and express a reduced amount of quail protein (Mahajan-Miklos, 1995).

Table 3. Alleles of *singed* used in this study

Allele	Allele strength	Bristle defect	Fertility	Mutation site
sn^2	weak	bent	fertile	polymorphism in 5' untranslated region
sn^4	weak	bent	fertile	unknown
sn^3	interm	gnarled	fertile	promoter deletion, cryptic promoter used
sn^{36a}	strong	gnarled	sterile	insertion, 5' untranslated region
sn^{X2}	null	gnarled	sterile	unknown
sn^{G409E}	interm	multiple bends	fertile, semi-dumpless	GLY 409 to GLU
sn^{S289N}	strong	gnarled	sterile	SER 289 to ASN
$sn^{SupS251F}$	interm	bent	fertile	SER 289 to ASN and SER 251 to PHE

Fertility was assessed by placing 10 females and 5 males in a vial. Vials were analyzed for larvae or pupae after 10 days and for adults after 17 days. Flies were considered sterile if no larvae or pupae were seen after 10 days. Flies were considered semifertile if they produced 40- 50% of the progeny produced by wild type females. Flies were considered weakly fertile if they produced less than 25% of the progeny produced by wild type females.

Antibody production

The *singed* open reading frame of 1536 base pairs was cloned in-frame adjacent to the carboxy-terminal segment of *Schistosoma japonicum* glutathione S-transferase (GST) in the expression vector pGEX-2T (Amrad corp., Melbourne, Victoria, Australia;(Smith and Johnson, 1988). This construct allows IPTG induction of a GST-singed fusion protein in *E. coli.* Fusion protein expression was induced with 1 mM IPTG for 4 hours at 37° C. Due to the highly insoluble nature of this fusion protein, it was isolated from inclusion bodies, electrophoresed on preparative 8% SDS-polyacrylamide gels, and identified by 0.3 M $CuSO_4$ stain. The fusion protein band was excised and the protein extracted by incubating the gel slice overnight in elution buffer (50 mM Tris pH 8, 0.1 mM EDTA, 0.2 mM NaCl, 0.1% SDS, 5 mM DTT). Purified protein was used to immunize mice. Antisera were screened for reactivity with the fusion protein by western immuno-blot and for immunoreactivity to *Drosophila* ovaries by western immuno-blot and immunofluorescence.

An immunoreactive mouse was used for hybridoma cell line production. Monoclonal cell lines were screened by ELISA, western immuno-blot, and immunofluorescence. Purification of IgG was carried out using HiTrap Protein G (Pharmacia, Piscataway, NJ).

Western immuno-blotting

Drosophila ovary extracts were obtained by grinding ovaries in Laemmli sample buffer and boiling for 5 minutes. Protein concentration was determined using BioRad's (Hercules, CA) modified Bradford assay. Extracts were separated by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes (Towbin et al., 1979). Following the transfer, the nitrocellulose membrane was blocked with Blotto (5% powdered milk and 0.1% Tween 20 in PBS) for 2 hours at room temperature. The membrane was then incubated overnight with monoclonal antibody supernatant diluted 1:10 in Blotto. After washing the membrane for one hour in PBS-Tween 20 (0.1%), the membrane was incubated with HRP-conjugated goat anti-mouse IgG secondary antibody (Pierce catalog #31434, Rockford, IL) at a 1/10,000 dilution in Blotto for 1 hour at room temperature. After four 15 minute washes with PBS-Tween (0.1%), the signals were detected using ECL western immuno-blot detection reagents (Amersham, Arlington Heights, IL) following the instructions of the manufacturer. Scanning densitometry was used to quantitate the signal intensity.

2D-Gel electrophoresis

For isoelectric focusing I used the O-Farrell method (O'Farrell, 1975). IEF gels were prepared using 3.2 % ampholytes pH 6-8 (Bio-Rad) and 0.8 % ampholytes pH 3-10 (Bio-Rad). Gels were cast in 11.5 cm x 3 cm diameter IEF tubes and overlaid with 8M urea.

30 ovaries were ground in 100 µl of sonication buffer with RNase and DNase and the sample was set on ice for 5 minutes. Solid urea was added to bring the sample to 9M urea (1 mg urea/µl sample). An equal volume of IEF Lysis buffer was added.

IEF gels were pre-focused at 500 volts for one hour. 100 µl of ovary extract (approximately 200 µg total protein) was loaded onto the IEF gels. Samples were overlaid with 80% lysis buffer and electrophoresed at 800 volts for 18 hours and then 1800 volts for 20 minutes. IEF gels were equilibrated for 10 minutes in SDS sample buffer and placed on top of standard 10 % SDS-PAGE gels for the second dimension electrophoresis (Laemmli, 1970). Transfer to nitrocellulose was performed according to Towbin (1979). Nitrocellulose was immunoblotted with singed monoclonal antibody 7C as described previously.

Northern analysis

3 flies were ground in 300 µl of RNA extraction buffer (0.1 M NaCl, 0.5 % SDS, 50 mM Tris pH 8, 10 mM EDTA, 50 µg/ml proteinase K) and incubated for 1 hour at 37° C. Samples were then extracted 2 times with phenol-chloroform (1:1) and once with chloroform. RNA was ethanol precipitated and resuspended 4.5 µl 1% diethylpyrocarbonate-treated (DEPC) water. RNA formaldehyde denaturation and gel electrophoresis were performed as described in Xue and Cooley(Xue and Cooley, 1993). RNA gels were blotted by capillary transfer onto Hybond-N (Amersham) and ultraviolet cross-linked to the membrane (Stratalinker, Stratagene). ^{32}P-labeled probes were prepared using random hexamer priming (Feinberg and Vogelstein, 1983) of the 1.5 kb *singed* open reading frame and the open reading frame of RPA as a loading control (Qian et al., 1987). Filters were hybridized in Church's buffer at 65° C with agitation (Church and Gilbert, 1984).

Egg chamber staining procedure

3-5 day old females were fed a water yeast paste for 24 hours. Ovaries were dissected and separated into individual egg chambers in ice-cold *Drosophila* EBR saline solution (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10

mM HEPES [pH 6.9]). Egg chambers were fixed in 100 µl of devitellinizing buffer (6% formaldehyde, 16.7 mM KH_2PO_4/K_2HPO_4 [pH6.9], 75 mM KCl, 25 mM NaCl, 3.3 mM $MgCl_2$) and 600 µl of heptane for 10 minutes with gentle agitation. Egg chambers were then washed extensively with PBT (1x PBS, 0.3% triton X-100, 0.5% BSA).

For actin staining, 5 µl rhodamine-conjugated phalloidin (Molecular Probes #R415, Eugene, OR) was vacuum dried and resuspended in 100 µl of PBT. Fixed egg chambers were incubated in rhodamine-conjugated phalloidin solution for 20 minutes in the dark and then were rinsed extensively in PBT. If nuclear staining was also desired, phalloidin-stained egg chambers were incubated in 1 µg/ml DAPI for 5 minutes. After washing egg chambers thoroughly in PBS, they were equilibrated in 50% PBS/glycerol and mounted onto slides.

For antibody staining, fixed egg chambers were blocked for 10 minutes in PBT. Egg chambers were incubated overnight at 4° C in 12 µg/ml purified anti-singed IgG. Egg chambers were washed extensively in PBT for one hour and then were incubated for 2 hours at room temperature in FITC conjugated goat anti-mouse IgG secondary antibody diluted 1/200 in PBT. Egg chambers were washed extensively in PBT for one hour, rinsed in PBS, and mounted in 50% PBS/glycerol.

Bristle staining

White prepupae were collected and dissected at 40-44 hours of pupal development according to Schweisguth and Posakony (Schweisguth and Posakony, 1992). The dorsal epithelial tissue was fixed in 4% paraformaldehyde for 30 minutes and then rinsed extensively in PBT for 2 hours. Tissue was then incubated for 20 minutes in either rhodamine-conjugated phalloidin (5 µl/100 µl PBT), 50 µg/ml unconjugated phalloidin

to stabilize actin filaments without fluorescent labeling, or no phalloidin as control. After rinsing the tissue for 30 minutes in PBT, it was incubated overnight at 4 °C in 12 μg/ml purified anti-singed IgG. The tissue was rinsed extensively for 1 hour in PBT and incubated for 3 hours at room temperature in FITC conjugated goat anti-mouse IgG secondary antibody diluted 1/200 in PBT. Tissue was washed extensively in PBT for one hour, rinsed in PBS, and mounted in 50% PBS/glycerol. Antibody staining was not affected by the presence or absence of phalloidin.

Singed protein production and purification

The *singed* open reading frame was cloned into the pET 17b expression vector (Novagen, Inc., Madison, WI). This construct allows bacterial expression of the entire singed peptide sequence with an additional 6 histidine residues followed by a thrombin cleavage site at the amino-terminus. To optimize solubility of the fusion protein, transformed bacteria were induced at 25° C with 0.05 mM IPTG for 3 hours. Soluble protein extracts in 0.1% Triton X-100, 5 mM imidazole, 0.5 M NaCl, 160 mM Tris-HCl, pH 7.9, were incubated with Ni^{2+} charged beads for one hour to bind the singed protein. These beads were then used to make a column that was extensively washed with a 60mM imidazole buffer containing 0.5 M NaCl, 20 mM Tris pH 7.9. Purified singed protein with the N-terminal histidine tag was eluted in 150 mM imidazole, 0.5 M NaCl, 20 mM Tris, pH 7.9. DTT (0.1 mM) was immediately added to eluted protein to prevent precipitation. Singed protein was dialyzed overnight into thrombin cleavage buffer, 20 mM Tris pH 8.4, 150 mM KCl, 1 mM DTT at room temperature. $CaCl_2$ was added to dialyzed protein to bring the solution to 2.5 mM $CaCl_2$. To remove the N-terminal histidine tag, thrombin (Novagen) was added at 1 unit/μg protein and incubated at room temperature for 2 hours. Following thrombin

cleavage, the purified singed protein was incubated again with Ni^{2+} charged beads for 30 minutes. The flow through containing purified singed protein was collected and any uncleaved His-tagged singed protein should remain bound to the column. Purified singed protein was dialyzed into storage buffer (20 mM Tris pH 8.4, 90 mM KCl, 0.1mM EDTA, 0.1mM DTT) and stored on ice. Approximately 1-3 mg of protein was obtained from a 200 ml bacterial culture. Purified protein remained soluble at 4° C for approximately 10 days. Singed solubility appeared to be sensitive to oxidizing conditions, therefore DTT was added every 3 days to prevent precipitation of protein. Protein was stable for 1 week at 4° C.

Low speed cosedimentation assay with singed and actin

G-actin was purified from chicken skeletal muscle acetone powder according to (Spudich and Watt, 1971). G-actin was polymerized in polymerization buffer (75 mM KCl, 2.5 mM $MgSO_4$, 10 mM imidazole, 10 mM EGTA, 0.1% NaN_3, pH 7.3). The approximate singed:actin ratio required to achieve apparent saturation of binding was determined by adding a range of purified singed (0.87 mM - 14 mM based on a molecular weight of 57 kD) to a fixed concentration of F-actin (7 mM, based on a molecular weight of 43 kD, stabilized as filaments with a 1:1 molar ratio of phalloidin) in a final solution of 85 mM KCl, 10 mM imidazole, 1 mM EDTA, 2.5 mM $MgSO_4$ pH 8.0. Samples were incubated at room temperature for one hour and then centrifuged 15 minutes at 16,000 g in a eppendorf microfuge. The relative distribution of actin and singed in the pellet and supernatant fractions was assessed by SDS-PAGE (8-15% gradient gels) and protein bands were visualized with coomassie stain. Density of protein bands was quantitated using scanning densitometry (BioRad transmittance scanning densitometer, Hercules, CA). Saturation was determined as the point at which singed:actin

protein ratio in the pellet remained constant. The molar ratio of singed to actin in the pellet fraction was graphed as a function of total singed protein concentration.

We tested the supernatant and pellet fractions of singed protein for actin bundling activity. A mixture containing 1:3 molar ratio of singed to actin was sedimented as above. More than 98% of the actin sedimented whereas the majority of singed remained soluble. F-actin (14 μM) was added to this soluble fraction of singed, incubated for 1 hour at room temperature, and centrifuged. The pellet and supernatant fractions from both the original sedimentation and the sedimentation with the soluble singed fraction were analyzed by SDS-PAGE.

To test bundling ability of singed recovered form a singed-actin pellet, singed:actin bundles were sedimented by centrifugation of a mixture containing 1:2 molar ratio singed:actin. The pellet fraction of singed-actin bundles was dissolved in 0.5 M KCl to dissociate singed. The F-actin was removed by high speed centrifugation (23 psi in Beckman airfuge for one hour) while the singed remained soluble. The recovered singed was then dialyzed into singed storage buffer and the concentration of singed was determined. Both the singed recovered from bundles and the original singed preparation were used to make mixtures of 1:2 molar ratio of singed:actin. These mixtures were sedimented and the supernatant and pellet fractions were analyzed by SDS-PAGE.

Microscopy

Fluorescent microscopy was carried out on a Zeiss Axiophot equipped with differential interference contrast and epifluorescence optics.

Confocal imaging was performed using a BioRad MRC 600 scanning laser confocal microscope. Optical sections were presented individually or were combined using the Comos software package (BioRad, Hercules, CA).

Scanning electron microscopy was performed on a ISI model SS-40 scanning electron microscope. Flies were dehydrated in isoamyl alcohol and mounted on stubs.

Mixtures of F-actin and singed in a 1:2 ratio were visualized by darkfield light microscopy and electron microscopy. Light microscopy was performed on a Zeiss light microscope with a darkfield condenser. Samples for electron microscopy were stained with 0.2% aqueous uranyl acetate on parlodion and carbon-coated grids and examined on a Zeiss 10CA electron microscope at an accelerating voltage of 80 kV.

Mutagenesis

Ethyl Methane Sulfonate (EMS) mutagenesis was carried out essentially as described (Lewis and Bacher, 1968).

Screen for new *singed* alleles: 0-4 day old isogenic w^{1118} males were starved for 12-16 hours and then fed 20 mM EMS in 1% sucrose for 16 hours. Flies were distributed into bottles with 10 mutagenized males and 40 *C(1)y f/Y* females. The flies were transferred to new bottles on day 2, 4, and 6. All males were removed prior to the final transfer on day 6 and the females were removed on day 8. All F1 males with bristle defects were selected and mated with *C(1)y f/Y* females. Independent lines were generated from males that could stably transmit the bristle defect to their progeny. Mutagenized X chromosomes were maintained in males by mating to *C(1)y f/Y* females. Complementation testing with *sn* and forked (*f*) was performed using *FM7c* sn^{X2} and f^{36a} chromosomes, respectively. Those bristle mutants that complemented *sn* and *f* were placed in complementation groups. Female

stocks were established by mating male bristle mutants with *FM7a* balancer females. Female homozygous for bristle defects were tested for fertility by placing 20 females and 10 males in a vial and looking for progeny after 10 days.

Screen for dominant suppressors of *sn* sterility: $w^{1118}\ sn^{S289N}/Y$ males were fed 25 mM EMS in 1% sucrose for 16 hours. Flies were distributed into bottles either at 25° C or 18° C. Each bottle contained 10 mutagenized males and 35 $w^{1118}\ sn^{S289N}/\ FM7a$ balancer females. The flies were transferred to new bottles on day 2. On day 4, the males were removed and the females were placed in new bottles and then removed on day 7. Homozygous $w^{1118}\ sn^{S289N}/w^{1118}\ sn^{S289N}$ F1 females were selected and tested for fertility. Any progeny obtained must have been derived from females carrying a dominant suppressor of the *sn* female sterility. F2 $w^{1118}\ sn^{S289N}/w^{1118}\ sn^{S289N}$ females were tested for fertility at 18° C by mating to fresh $w^{1118}\ sn^{S289N}/Y$ males. Any F2 $w^{1118}\ sn^{S289N}/Y$ males obtained were mated with stock $w^{1118}\ sn^{S289N}/FM7a$ females and the subsequent F3 $w^{1118}\ sn^{S289N}/w^{1118}\ sn^{S289N}$ females were tested for fertility at 18° C.

Molecular analysis of *sn* alleles

All basic cloning techniques were performed as described in Sambrook *et. al.* (Sambrook et al., 1989). Genomic DNA was prepared from homozygous *sn* flies by standard techniques (protocol 48 in (Ashburner, 1989b). *sn* exons were amplified from genomic DNA using PCR. PCR primers were designed to allow amplification of exon 2 and exons 3-6. PCR fragments were purified using PCR Magic Preps (Promega) or gel purified using Qiaex gel extraction kit (Qiagen). 5' overhangs on PCR products were filled in using Klenow DNA polymerase (New England BioLabs). Blunt PCR products were subcloned into the EcoRV or the Sma1 site of PCRscript (Statagene). Blue-white color

selection was used to identify plasmids containing inserts. Insert-containing plasmids were sequenced using the USB sequenase kit. All mutations were confirmed by sequencing clones from independent PCR reactions.

Chapter 3

UNDERSTANDING THE *SINGED* PHENOTYPES

INTRODUCTION

Depending on the allele, *singed* (*sn*) bristles vary from short and gnarled to wavy and bent. In severe mutants, large bristles (macrochaetes), small bristles (microchaetes), and hairs on the head, thorax, legs and wings are all affected to varying degrees (Figure 4A, B). The morphology of the bristle appears to reflect the organization and integrity of the cytoskeletal core present at the time of cuticle deposition during bristle development. Aberrant bristle morphology has been correlated with defects in the organization and composition of actin filament bundles in *sn* bristles (Overton, 1967). Electron microscopy analysis of gnarled sn^3 bristles showed that the microtubule component of the developing shaft appeared normal; however, the actin filament bundles appeared very small, flat and ribbon-like rather than round and columnar. This decrease in size of the actin filament bundles suggested that *sn* mutants may have a defect in actin organization, such as in actin bundle formation.

The severity of the bristle defect generally correlates with the *sn* female sterile phenotype such that *sn* mutants with gnarled bristles are also female sterile. Severe *sn* females are sterile due to a defect in oogenesis (Bender, 1960; Gutzeit and Strauß, 1989). In sterile *sn* mutants, oogenesis becomes defective at the onset of rapid cytoplasm transport (Gutzeit and Strauß, 1989). Egg chambers of sterile *sn* mutants appear normal prior to rapid cytoplasm transport but subsequently, the nurse cells fail to regress leading to the formation of small eggs (Figure 4C, D). The follicle cells, however, continue their developmental program and synthesize all the components of the egg

Figure 4. *singed*^{X2} mutants have defective bristle development and oogenesis

Scanning electron micrographs are shown of heads from wild type (A) and *sn*^{X2} flies (B). Wild-type bristles are long and straight (arrow in A) while *sn* bristles are bent and curved with a spiral-like or branched appearance (arrow in B). Mature eggs from wild type (C) and sterile *sn*^{X2} females (D) differ in size. Dorsal appendages and egg shell chorion have formed on both eggs yet the *sn*^{X2} egg appears half the size of the wild type egg and the nurse cells fail to regress.

shell [Bender, 1960 #468; Gutzeit, 1989 #420]. The follicle cell-derived structures appear affected in sterile *sn* mutants; respiratory appendages are often flattened and fused and the operculum forms at almost a right angle to the long axis of the egg. These defects are likely to be secondary consequences of the failure of nurse cell regression (Gutzeit and Strauß, 1989).

The *sn* oogenesis phenotype is very similar to the phenotype of *chickadee* (Cooley et al., 1992b) and *quail* (Mahajan-Miklos and Cooley, 1994b). Both *chickadee* and *quail* encode proteins with homology to actin binding proteins and both are required for the formation of nurse cell cytoplasmic actin filament bundles. *chickadee* encodes *Drosophila* profilin and *quail* encodes a villin-like protein. In addition to similarities in oogenesis phenotype, severe *chickadee* mutants also have a bristle defect (Verheyen and Cooley, 1994).

The reduction in actin filament bundles in *sn* bristles and the failure of nurse cell rapid cytoplasm transport in egg chambers of sterile *sn* females both suggest that the singed protein may interact with the actin-based cytoskeleton. In this chapter, I show that singed protein is expressed in the cytoplasm of a variety of cell types and is required for the formation of actin filament bundles in nurse cells and developing bristles. This phenotypic defect is consistent with the sequence homology described between singed and the actin bundling protein fascin.

RESULTS

Filamentous actin in singed egg chambers

Most *sn* mutants with severe bristle defects are female sterile and produce egg chambers in which the final rapid phase of cytoplasm transport to the oocyte is incomplete. Since this process is actin-dependent (Gutzeit,

1986a), I investigated actin distribution in *sn* egg chambers. Actin filaments are normally present subcortically in nurse cells, including the ring canals connecting adjacent cells (Figure 5A). Subcortical actin-containing filaments likely support myosin-based nurse cell contraction to push nurse cell cytoplasm through the ring canals into the oocyte (Cooley et al., 1992b; Gutzeit, 1986a; Wheatley et al., 1995). In late stage 10, just prior to the rapid phase of cytoplasm transport, actin filament bundles form in the nurse cell cytoplasm (Figure 5A); these bundles probably have a structural role in anchoring the nurse cell nuclei in a central position away from ring canals (Cooley et al., 1992a). I analyzed actin-based structures using rhodamine-phalloidin to stain F-actin in egg chambers from sterile *sn* mutants. *sn* mutant egg chambers were indistinguishable from wild type prior to the rapid phase of nurse cell cytoplasm transport. However, in egg chambers from sterile *sn* mutants, the cytoplasmic actin filament bundles rarely formed (Figure 5B) and nurse cell nuclei became dramatically rearranged (Figure 5C, D). The nuclei in the four nurse cells adjacent to the oocyte appeared to be pushed into the ring canals, to extend into the oocyte (Figure 5D), and to block the flow of cytoplasm into the oocyte. Some nuclei also appeared pushed into ring canals between adjacent nurse cells (not shown).

Production of singed monoclonal antibodies

To analyze singed protein expression in wild type and *sn* mutants, I made monoclonal antibodies from a mouse immunized with a GST-singed fusion protein. These monoclonal antibodies recognized a 57 kD protein by western immuno-blot in whole fly extracts of males and females (not shown) and in ovary extracts (Figure 6). *sn* mutants also contained a 57 kD protein; however, the level of singed protein was reduced. To quantitate levels of singed protein in wild type and *sn* ovaries, 25 μg of protein extract was loaded

Figure 5. Absence of nurse cell cytoplasmic actin filament bundles and subsequent nuclear rearrangement in egg chambers from sterile *singed*^{X2} mutants

Stage 10B egg chambers from wild type (A, B) and *sn*^{X2} egg chambers (C, D) were double stained with rhodamine-conjugated phalloidin (A, C) and DAPI (B, D). In wild type, cytoplasmic actin bundles (cb) form during late stage 10 (A) and the nurse cell nuclei are located centrally in the nurse cell (B). In *sn*^{X2} mutants, cytoplasmic actin filament bundles are rare (C) and nurse cell nuclei become rearranged and appear to be pushed into ring canals (arrowheads in D). Subcortical actin filaments (sc) and ring canals (rc) appear unaffected in mutants. In the DAPI stained wild type egg chamber (B) the small nuclei of the follicle cells (fc) are seen in the surface focal plane.

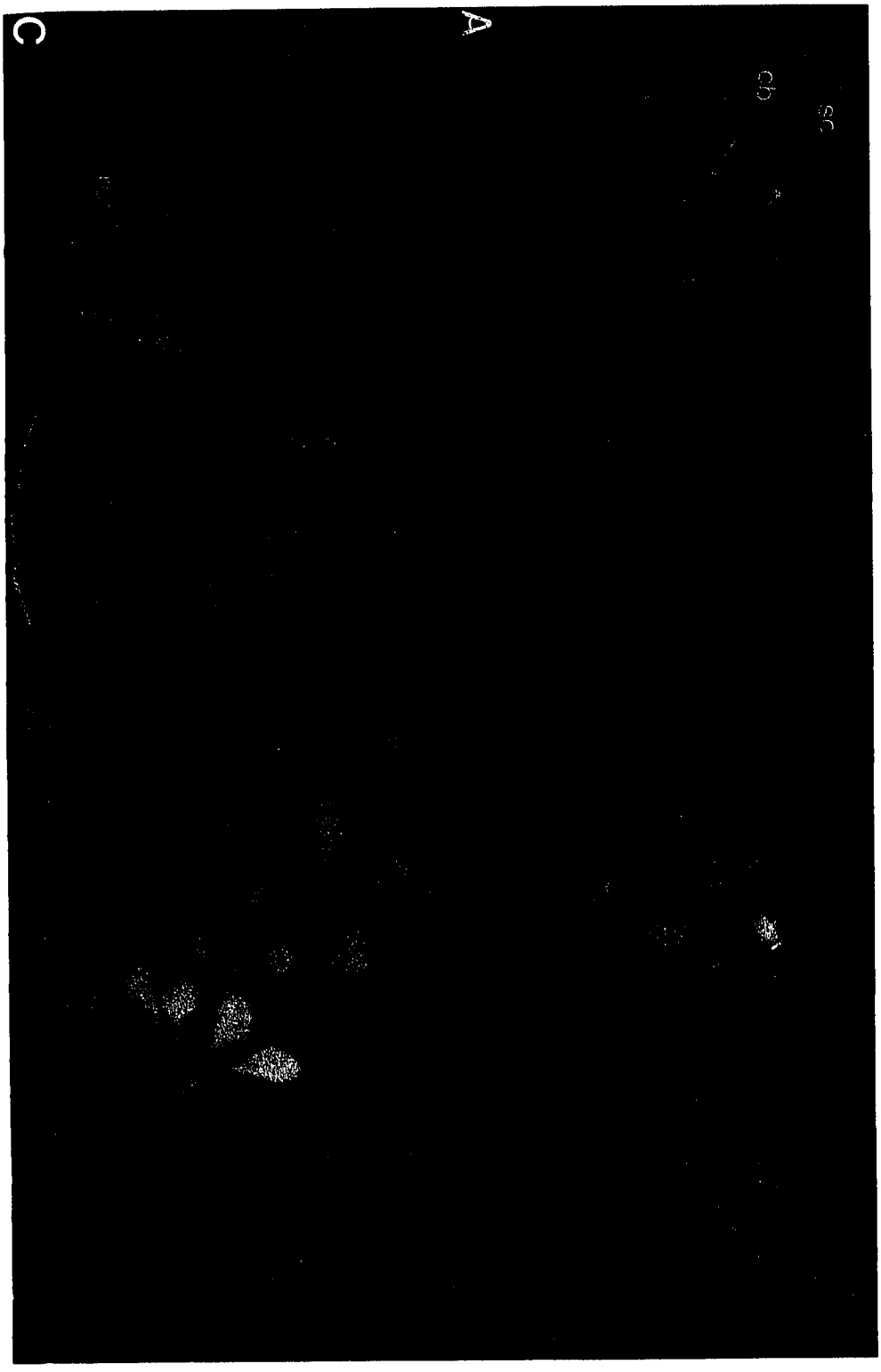
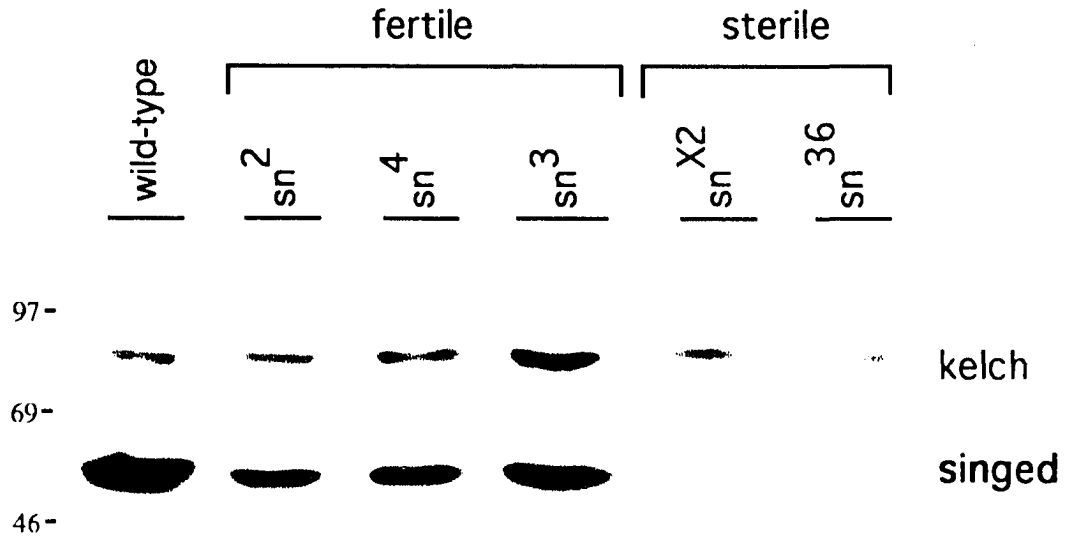


Figure 6. Singed monoclonal antibody recognizes a 57 kD protein

Western immuno-blot analysis of singed protein expression in ovary extracts from wild type and fertile and sterile *sn* mutants was performed. 25 μg of total protein was loaded per lane. The blot was probed with singed monoclonal antibody 7C and kelch monoclonal antibody 1B (Xue and Cooley, 1993). The singed monoclonal antibody recognized a single 57 kD protein that is reduced in fertile *sn* mutants and absent or nearly absent in female sterile *sn* mutants. Kelch monoclonal antibody recognized an 80 kD protein and was used as a loading control. Protein molecular weight markers are indicated in kD on the left edge of the gel.



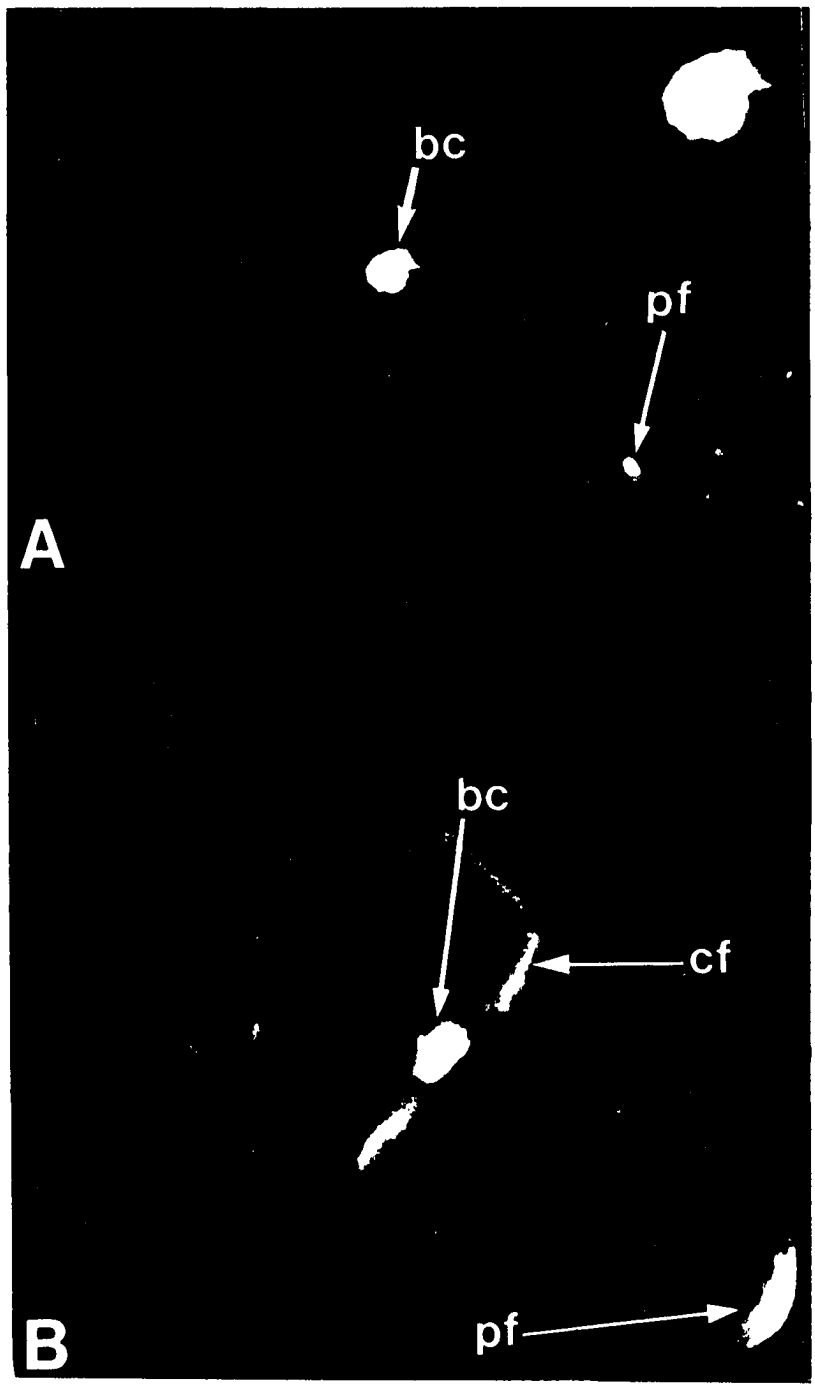
in each lane and kelch antibody was used as a loading control (Xue and Cooley, 1993). Fertile *sn* alleles, sn^2, sn^3, and sn^4, contained markedly reduced levels of the 57 kD protein relative to wild type levels as determined by densitometric scanning of autoradiograms like the one shown in Figure 6. In sterile *sn* mutants, sn^{X2} and sn^{36a}, the 57 kD protein was absent or nearly absent. Three transcripts are generated from the *sn* locus that differ only in the 3' untranslated region and encode the same 57 kD protein (Paterson and O'Hare, 1991). The absence of protein in severe, sterile *sn* alleles suggested that all transcripts are affected. The increased severity of the mutant phenotype appeared to correlate with the decreased amount of singed protein detected.

Singed protein localization in egg chambers

I stained egg chambers from wild type and both fertile sn^3 and sterile sn^{X2} mutants with singed monoclonal antibody purified IgG. In the germarium and subsequent early stages of oogenesis in wild type females, singed was detected at low levels in nurse cell cytoplasm. Several migratory populations of follicle cells expressed very high levels of singed. At stage 9, abundant staining was present in border cells and posterior follicle cells (Figure 7A). The border cells formed spike-like projections visible by anti-singed immunofluorescence as they migrated between nurse cells from their anterior position in the egg chamber to the anterior margin of the oocyte (Figure 7A, B). As border cells migrate between nurse cells, the majority of follicle cells surrounding the egg chamber migrate posteriorly along the outside of the egg chamber to form a columnar epithelium around the oocyte. A small number of follicle cells remain, surrounding the nurse cells in a squamous epithelium. The follicle cells migrating around the outside of the egg chamber do not express abundant singed protein (Figure 7A). In early

Figure 7. Localization of singed protein in migratory follicle cells in egg chambers

Wild-type egg chambers were stained with purified singed monoclonal antibody IgG (12 μg/ml) and examined using confocal microscopy. In stage 9 egg chambers, singed is abundantly expressed in the cytoplasm of border cells (bc) and posterior follicle cells (pf) (A). As these cells migrate between nurse cells toward the anterior margin of the oocyte, singed protein is also detected in several spike-like cellular extensions (inset). In early stage 10 egg chambers (B), the border cells t the anterior margin of the oocyte, the posterior follicle cells, and centripetal follicle cells (cf) all contain abundant singed protein. As centripetal follicle cells migrate along the nurse cell oocyte interface, singed protein appears to have a subcellular localization near the follicle cell-nurse cell interface. These image projections represent the combination of serial confocal optical sections.

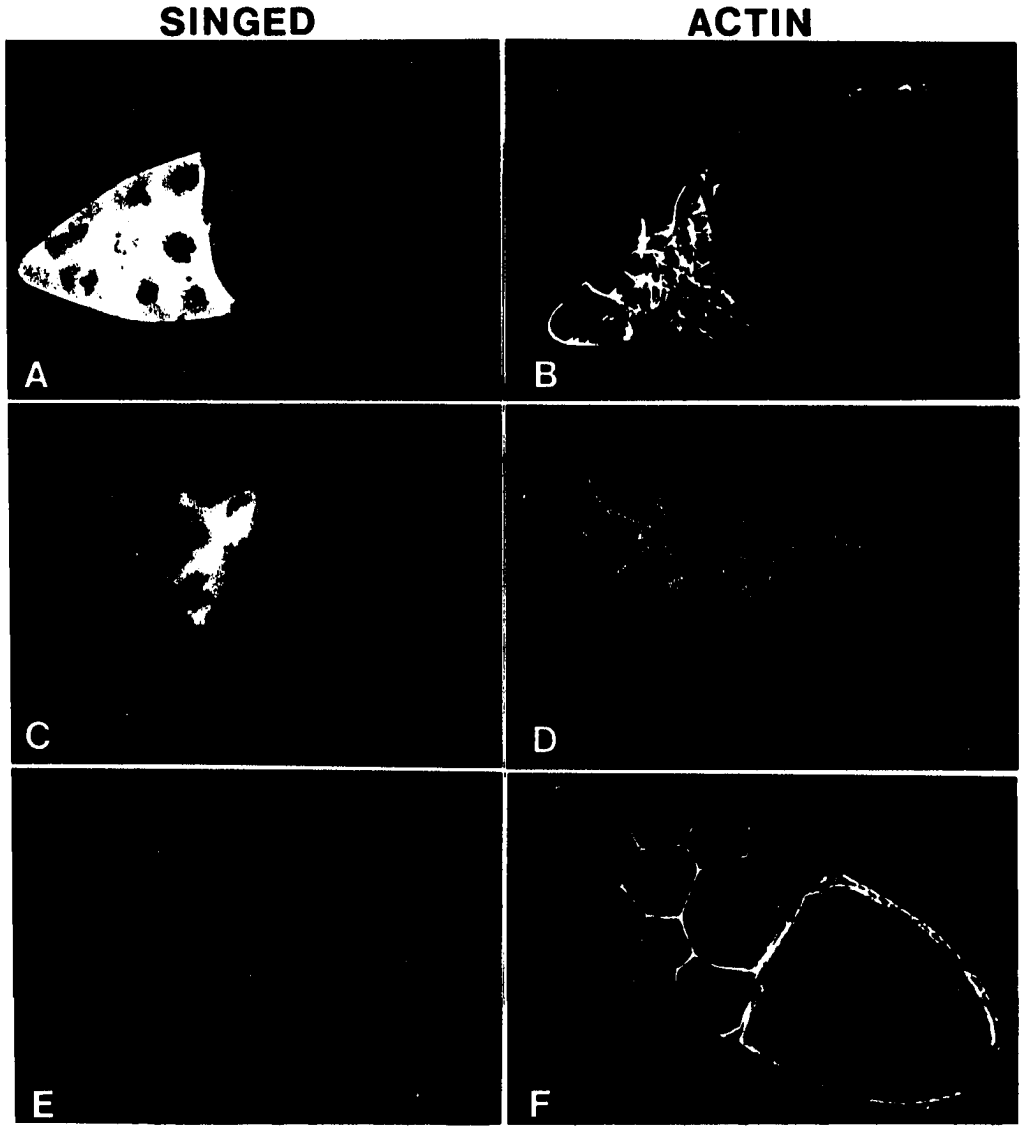


stage 10, singed was expressed abundantly in the centripetal follicle cells as they migrated along the nurse cell-oocyte interface (Figure 7B). Singed expression in these cells appeared localized near the follicle cell-nurse cell interface. These data suggest that only the follicle cells that migrate to the interior of the egg chamber express abundant singed protein.

During stage 10 there was a dramatic increase in singed expression in the nurse cells. Nurse cell cytoplasm showed a low concentration of singed protein in early stage 10 egg chambers (Figure 7B) and a high concentration at the end of stage 10 when actin filament bundles form. In stage 11, during rapid nurse cell cytoplasm transport, singed protein staining continued to increase throughout the nurse cell cytoplasm and also intensified in subcortical regions (Figure 8A). Polyclonal sera and ten different monoclonal supernatants all demonstrated this staining pattern. Gentle extraction of egg chambers with 0.5% triton X-100 or saponin decreased the nurse cell cytoplasm staining intensity but did not alter the diffuse character of the staining pattern (not shown). In fertile sn^3 mutants, singed protein staining was slightly reduced, but the localization and character of the staining was not significantly affected (Figure 8C). The presence of singed protein in both wild type and fertile sn^3 mutants correlated with the presence of normal cytoplasmic actin filament bundles (Figure 8B, D). Singed protein was not detected in nurse cells, border cells, posterior follicle cells, or centripetal follicle cells in egg chambers from sterile sn^{X2} mutants (Figure 8E). The absence of protein in nurse cells in sterile sn^{X2} mutant egg chambers (Figure 8E) correlated with the near absence of cytoplasmic actin filament bundles (Figure 8F).

Figure 8. Singed expression is necessary for cytoplasmic actin bundle formation

Stage 11 egg chambers from wild type and fertile and sterile alleles of *sn* mutants were stained with either purified singed monoclonal antibody IgG (sn) or rhodamine-conjugated phalloidin (actin) during the rapid phase of nurse cell cytoplasm transport. In wild type, cytoplasmic actin filament bundles are present (B), and singed protein is expressed abundantly in the nurse cell cytoplasm (A). In egg chambers from fertile sn^3 females, singed protein expression is slightly reduced (C) but the actin filament structures appear normal (D). In egg chambers from sterile sn^{X2} females, singed protein is absent (E), and cytoplasmic actin filament bundles are nearly absent (F) while subcortical actin filaments appear normal.



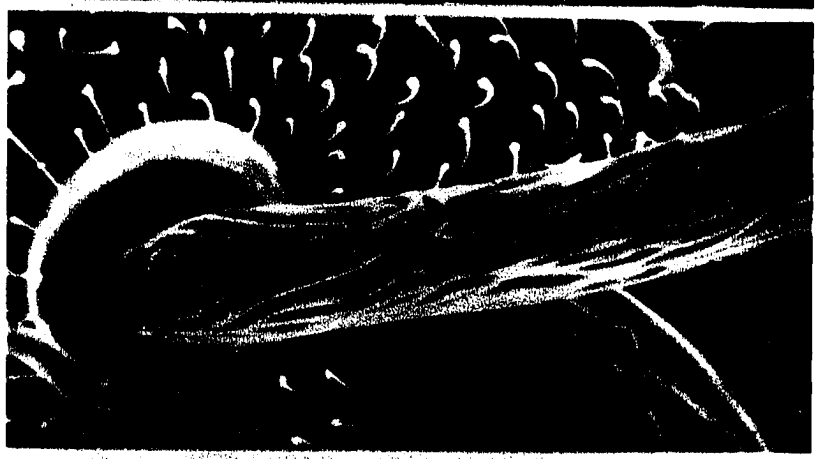
Filamentous actin and singed expression in bristles

In *Drosophila*, each bristle organ is comprised of four clonally derived cells: the trichogen, tormogen, nerve cell, and sheath cell. The bristle shaft forms from a single cytoplasmic extension of the trichogen cell. The extension is supported by 8-12 submembranous actin filament bundles and a central region of microtubules (Appel et al., 1993; Overton, 1967). Bristle elongation occurs between 30-45 hours of pupal development. External ridges and microfilament bundles are apparent at 35 hours, and the bristle has extended to 75% of its final length by 41 hours (Lees and Picken, 1945). Overton hypothesized that as the bristle is extending, the plasma membrane protrudes between adjacent actin filament bundles resulting in a ridged bristle surface during both bristle elongation in the pupae and in the adult after cuticle deposition. In the adult cuticle, ridges represent the outpatching of the cell membrane while grooves represent the position of submembranous actin filament bundles that were present during bristle development (Figure 9A). The tormogen cell forms the socket that surrounds the bristle shaft at its base. Final differentiation of the bristle includes innervation and disappearance of the cytoskeletal core.

I analyzed *sn* bristle structure in adults using scanning electron microscopy. Wild type bristles were straight with gradually tapering ends and parallel longitudinal ridges and grooves running the length of the bristle (Figure 4A, 9A). sn^{X2} bristles appeared to have an increased number of ridges that were not parallel and often intersected or fused with other ridges (Figure 9B). The scutellar bristles on sn^{X2} mutants were the most severely deformed; some regions of the shaft appeared collapsed and the bristles appeared spiraled, bent, and branched (Figure 4, 9C).

Figure 9. Aberrant bristle morphology in sn^{X2} mutants

Scanning electron microscopy images of adult bristles from wild type (A) and sn^{X2} mutants (B, C) are shown. Wild type bristles are straight with parallel longitudinal ridges and grooves in the cuticle. In sn^{X2} bristles, the ridges and grooves are highly irregular and often intersect or fuse (B). The scutellar sn^{X2} bristles are most deformed and appear curved, bent, branched and sometimes collapsed (C). A and B are the same magnification. Scale bars represents 10 μm.



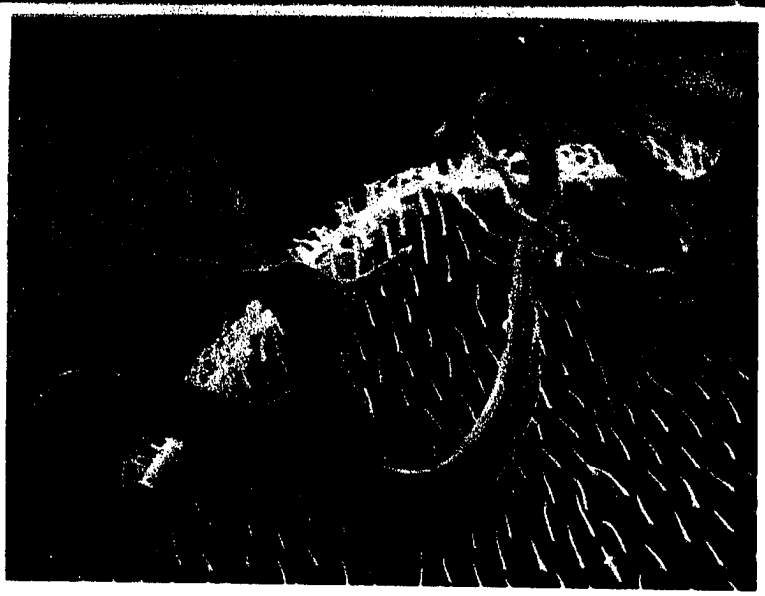
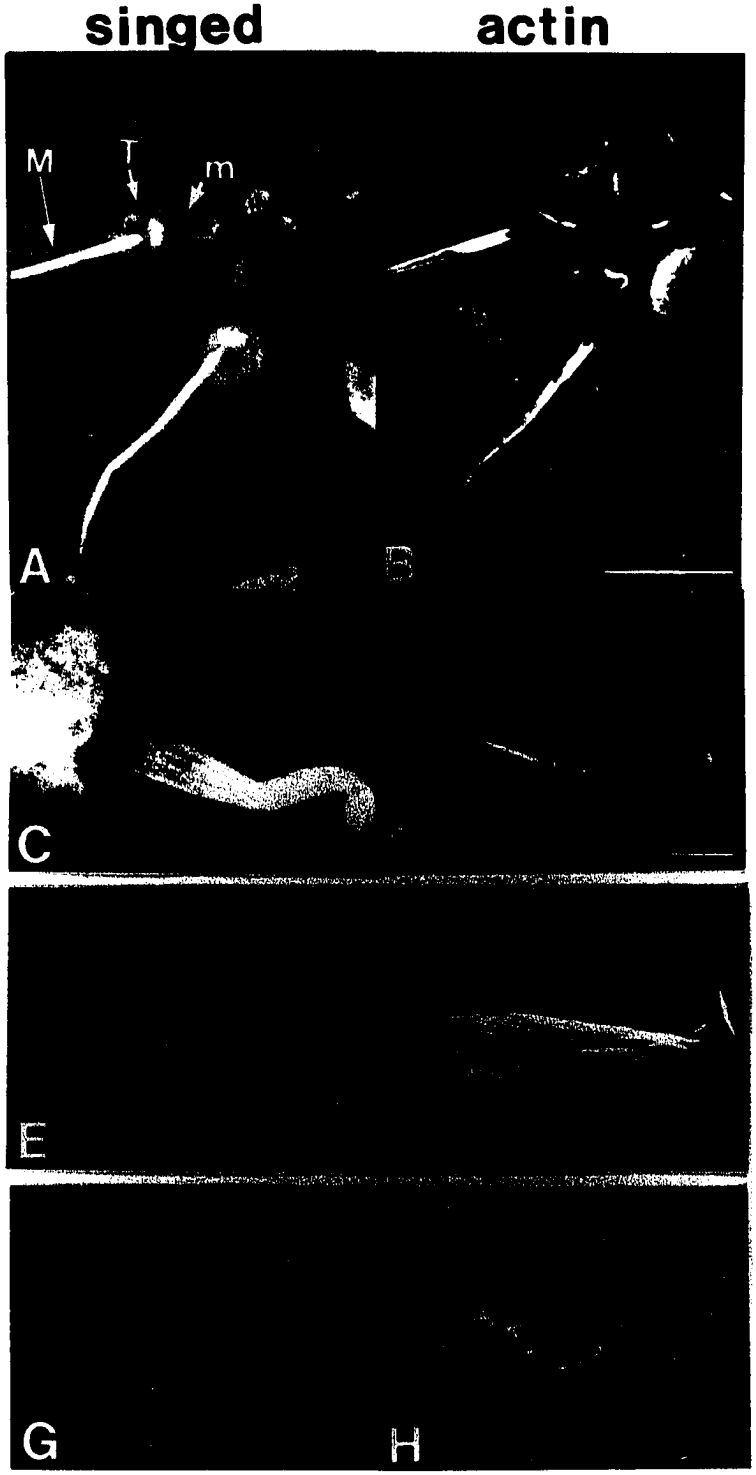


Figure 10. Absence of singed during bristle extension correlates with defective actin bundles

The dorsal epithelia of 40-44 hour pupae were double stained with purified singed monoclonal antibody IgG (A, C, E, G) and rhodamine-conjugated phalloidin (B, D, F, H). In wild type pupae (A, B, C, D), singed protein is expressed in the cytoplasm of the bristle extensions in both macrochaetes (M) and microchaetes (m) and in the tormogen socket cell (T). Wild type bristle extensions contain parallel actin filament bundles (B, D). In addition to the diffuse cytoplasmic localization of singed protein in the bristle extension, singed often appears more concentrated in a bundle-like pattern (C) coincident with submembranous actin filament bundles (D). In sn^3 bristles (E, F) singed protein is nearly absent and actin filament bundles are disorganized. In sn^{X2} bristles singed protein is also nearly absent (G) and the actin filament bundles appear diffuse and disorganized (H). The overall shape of the sn^{X2} bristle is curved and branched (H). Note that the rhodamine-conjugated phalloidin staining signal does not bleed into the FITC channel (E, G). C-H are the same magnification. Scale bars represents 10 μm.



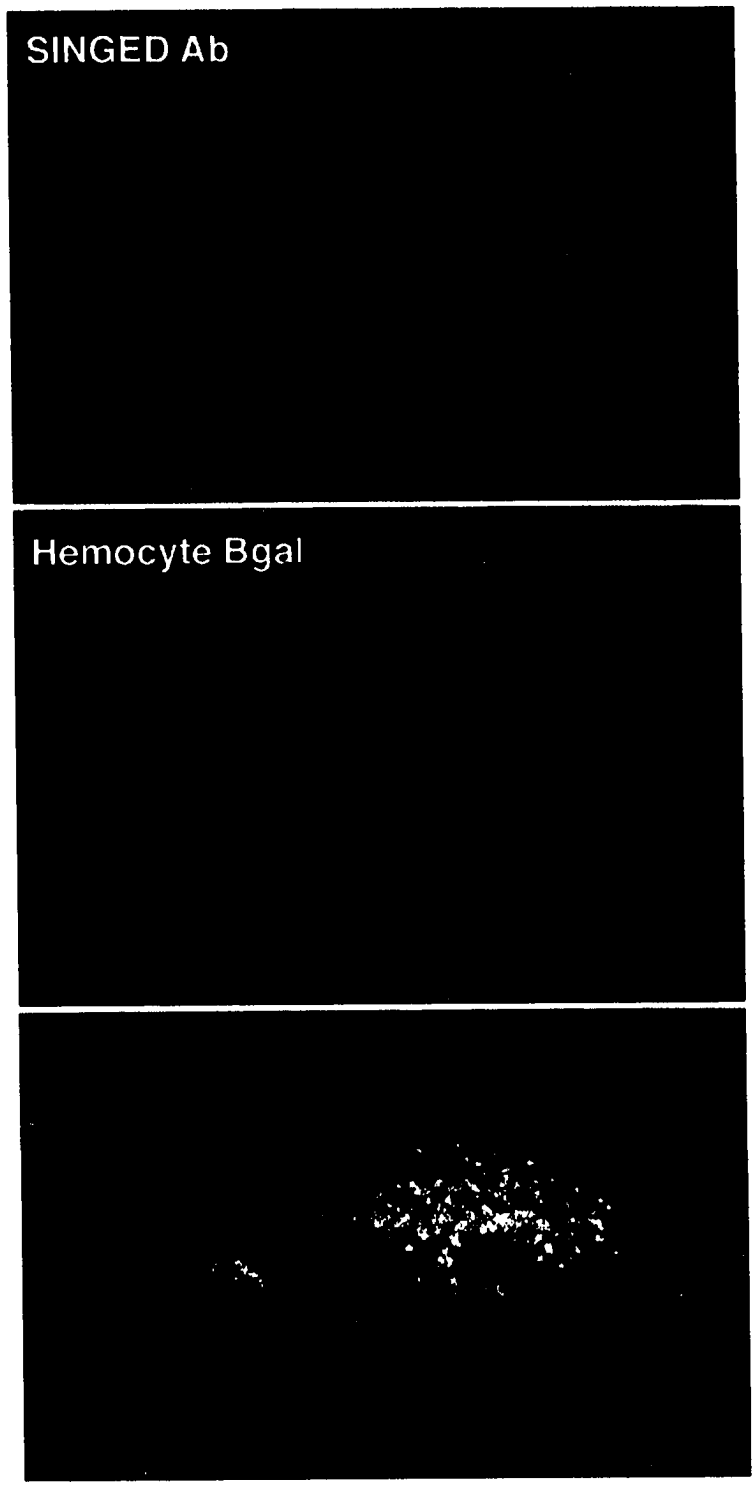
By examining the organization of actin filaments and the localization of singed protein in developing bristles, I found that singed appeared to be required to form organized actin filament bundles. In wild type pupae, singed protein was present in the cytoplasm of the extending bristle and also in the tormogen cell that forms the socket surrounding the base of the bristle shaft (Figure 10A, C). In about one-third of the bristles examined I also observed increased singed protein staining in a bundle-like pattern coincident with actin filament bundles (Figure 10C). In wild type bristles, rhodamine-conjugated phalloidin staining showed discrete and parallel actin bundles extending the length of the bristle (Figure 10B, D). Actin filaments were also present in the tormogen cell (not shown). Actin filaments and singed protein expression were then analyzed in bristles from sn^3 and sn^{X2} pupae. sn^3 mutants have moderately gnarled bristles, whereas sn^{X2}, a putative null allele, have the most severely gnarled bristles. In sn^3 bristles, singed protein was nearly absent and the actin filament bundles appeared disorganized (Figure 10E, F). In sn^{X2} bristles, singed protein was also absent, although the rhodamine-phalloidin staining of actin filament bundles appeared more diffuse, faint, and extremely disorganized (Figure 10G, H). Singed protein in sn^3 mutants may not be expressed at this stage of bristle extension or immunofluorescence may not be sensitive enough to detect differences in protein expression in sn^3 versus sn^{X2} bristles.

Singed is expressed in embryonic hemocytes

Dramatic actin cytoskeletal alterations accompany cellularization and gastrulation during embryogenesis. Embryos contain maternal *singed* mRNA. To determine if singed protein is expressed during embryogenesis, I stained 0-24 hour embryos with singed monoclonal antibody 7C. Singed is present in the cytoplasm of many cells throughout embryogenesis but is

Figure 11. Singed expression in embryonic hemocytes

A109 embryos contain a P[βgal] insert into the collagen 4 gene and express βgal in embryonic hemocytes. To determine if singed protein is expressed in embryonic hemocytes, A109 embryos were double labeled with singed mouse monoclonal antibody and rabbit anti-βgal antibodies. In this stage 14 embryo, FITC singed fluorescence is shown in green (top panel) and rhodamine βgal fluorescence is shown in red (middle panel). Cells that contain both βgal and singed are yellow in double labeled embryo shown in the bottom panel. In A109 embryos, both βgal and singed are expressed in hemocytes.



highly expressed in a subset of cells that resembled hemocytes (Figure 11). To confirm the presence of singed in hemocytes, I obtained a hemocyte enhancer trap line (A109) that had P[β-gal] inserted into the collagen IV promoter (Wilson et al., 1990). I doubled labeled embryos that express β-gal in the hemocyte nucleus with mouse singed monoclonal antibodies (11A) and rabbit β-gal antibodies (11B). In these embryos, singed and B-gal were expressed in the same cell (Figure 11C). The functional significance of singed in hemocytes is not clear. I was not able to examine embryos that did not contain maternal *singed* mRNA because null *singed* alleles are female sterile .

DISCUSSION

The phenotypes of *sn* mutants suggest that singed protein, like fascin, organizes actin filaments into bundles *in vivo*. Defective actin filament bundles in *sn* mutants can account for both the nurse cell cytoplasm transport arrest and the bristle deformity in *sn* mutants. Sterile sn^{X2} egg chambers contain no singed protein and nurse cell cytoplasmic actin filament bundles are nearly absent. The absence of cytoplasmic actin filament bundles allows the nurse cell nuclei to lodge into ring canals and block nurse cell rapid cytoplasm transport. In sn^{X2} bristles, the absence of singed protein correlated with both the faint, disorganized actin staining in developing bristles and the subsequent aberrant adult cuticle pattern. In wild type, dense actin filament bundle columns appear to promote bristle extension in a single direction possibly by providing structural support and by directing cytoplasmic forces toward the tip through symmetric and circumferential force opposition. In electron micrographs of wild type developing bristles, actin filament bundles have an approximately 12 nm cross-banding periodicity (Overton, 1967; Tilney et al., 1995) similar to *in vitro* singed-actin bundles (Chapter 4). In *sn*

mutants, a decrease in actin bundling protein may result in bundles that contain fewer actin filaments and lack the usual rigid parallel organization ensured by saturated cross-linking binding sites. The small defective actin bundles in singed mutants are no longer hexagonally packed (Tilney et al., 1995). I suggest that the thin, disorganized filament bundles in gnarled *sn* bristles cannot provide the structural support for continuous, straight extension. This decrease in structural integrity could allow actin filament bundles to bend or divide causing bristles to curve and branch. Lateral bending of actin filament bundles could explain the cross-weave cuticle ridge pattern seen with scanning electron microscopy and result in the spiral curvature of some *sn* bristles. Singed protein may affect the assembly and stabilization of actin filament bundles, the structural integrity of actin filament bundles, or actin monomer/polymer dynamics.

In the bristle extension, singed protein appears predominantly cytoplasmic, with an occasional diffuse, bundle-like pattern coincident with filamentous actin. In the nurse cells of egg chambers, singed protein appears diffusely cytoplasmic with an increased concentration along the nurse cell oocyte interface and subcortically during the rapid phase of nurse cell cytoplasm transport. I propose that singed protein bound to actin filament bundles is difficult to observe in the presence of a large excess of unbound protein. A potentially analogous situation has been found in sea urchin eggs in which most of the fascin is present in the cytoplasm as determined by fractionation assays (Otto et al., 1980). Upon fertilization, 30-35% of the fascin shifts to the pelletable fraction suggesting that only a portion of fascin is recruited to the newly formed microvillar cytoskeleton. Fascin associated with actin bundles is best visualized in isolated sea urchin egg cortex microvilli (Otto et al., 1980) and permeabilized coelomocyte filopodia

extensions (Otto and Bryan, 1980; Otto et al., 1979). In these preparations, unbound, cytoplasmic fascin would not be present.

Our phenotypic and biochemical data argue strongly that mutations in the fascin-encoding *sn* gene are responsible for all of the *sn* phenotypes observed. It is unlikely but formally possible that mutations affecting the *sn* gene also affect neighboring genes and that they contribute to the phenotypes. Transgenic experiments with copies of the cloned *sn* gene would test the ability of the fascin homolog to rescue *sn* mutants; however, such experiments have not yet been carried out.

Singed expression in cells undergoing dynamic cytoskeletal rearrangements

Singed appears to be abundantly expressed in specific subsets of migratory somatic follicle cells in egg chambers and embryonic hemocytes. The border cells migrate between the nurse cells to take up position at the nurse cell-oocyte interface. During their migration, cell extensions are clearly visible with singed immunofluorescence. Posterior follicle cells also express abundant singed. These cells have migratory potential although normally the oocyte blocks their path (Montell et al., 1992). As centripetal follicle cells migrate inward along the nurse cell-oocyte interface, they express high levels of singed. Therefore, specific migratory follicle cells of the egg chamber express singed abundantly.

Singed is expressed in embryonic cells that express collagen IV. Embryos that have a P[β-gal] inserted into the collagen IV promoter showed colocalization of singed and β-gal. Collagen IV has been shown to be expressed in hemocytes, a migratory, mesodermal cell population that functions to synthesize major hemolymph proteins, deposit basement membrane components, and to phagocytose autolysing or reforming cells (Mirre et al., 1988). Interestingly, in sea urchin, fascin is also expressed in

phagocytic cells called coelomocytes where it bundles actin filaments in filopodial extension (Otto and Bryan, 1980; Otto et al., 1979). There are no embryonic phenotypes associated with the null allele sn^{X2}, but specific functions of embryonic hemocytes have not been examined in the absence of singed.

The presence of singed in cells undergoing dynamic actin reorganization associated with migration is not surprising. Fascin has been localized to filopodial extensions that form rapidly on sea urchin coelomocytes. HeLa cell 55 kD protein (Yamashiro-Matsumura and Matsumura, 1986; Yamashiro-Matsumura and Matsumura, 1985), which was also recently shown to have homology with fascin peptide sequence (Bryan et al., 1993), immunolocalizes to highly motile microspikes and also stress fibers. However, sn^{X2} null mutants do not display any adult phenotypes that are reminiscent of defects in embryogenesis. Border cell and follicle cell migrations appear unaffected in sterile sn^{X2} mutants, even though no singed protein is detected in these cells. Additional actin bundling proteins could be present that can compensate for the absence of singed during cytoskeletal rearrangements associated with migration. A requirement for *sn* in egg chamber somatic cells remains unclear. Mosaic analysis has shown that females with *sn* mutant germline-derived cells and wild type somatic follicle cells exhibit a *sn* phenotype suggesting that *sn* is required in the germline (Perrimon and Gans, 1983). However, mosaic females with *sn* somatic cells and wild type germline-derived cells have not been described, and a somatic cell defect can not be excluded.

Aberrant actin bundle formation in *Drosophila* mutants with bristle defects

The highly organized, actin-based cytoskeletal framework in developing bristles requires multiple proteins. In addition to *sn*, several fly

bristle morphology mutants, including *forked* (Petersen et al., 1994), *chickadee* (Verheyen and Cooley, 1994), and *Stubble-stubbloid* (Appel et al., 1993), also have defective actin filament bundle structures (Table 1). The *forked* bristle phenotype most closely resembles the *sn* phenotype. The *forked* locus contains many transcripts, none of which encode proteins with homology to any known actin binding protein (Hoover et al., 1993). However, forked protein is localized on actin filament bundles in developing bristles prior to singed protein expression (Petersen et al., 1994) and in severe null *forked* alleles, actin filament bundles are very small but retain hexagonal packing (Tilney et al., 1995). In addition, overexpression of small *forked* transcripts disrupts the structural integrity of developing bristles (Petersen et al., 1994). High concentrations of bundling protein during the initial phase of bundle formation *in vitro* can result in rapid aberrant cross-linking of actin filaments without regard to order (Stokes and DeRosier, 1991). Forked may indeed be a novel actin binding protein that directly affects actin filament bundle organization and acts together with singed to provide highly ordered, rigid actin filament bundles.

In addition to *singed* and *forked*, *chickadee* and *Stubble-stubbloid* mutations have recently been shown to affect the actin filament bundles in bristles [reviewed in introduction; Appel, 1993 #427; Verheyen, 1994 #424]. The structural integrity of the developing bristle extension appears to require a wide array of proteins for the formation of organized, dense actin filament bundles.

CONCLUSIONS

I have found that singed protein is expressed in a variety of cells and that a deficiency of singed protein affects actin integrity in distinct cell types

differently, ranging from absence of actin bundles in nurse cells to disorganized actin bundles in bristles. In contrast, migratory cells in egg chambers, which have intense singed staining in wild type, appear unaffected in severe *sn* mutants. Cell types that appear less affected may contain additional cytoskeletal proteins that act redundantly with singed or can substitute for singed in severe *sn* mutants. In addition, the developmental time frame constraining actin rearrangement in a given cell probably influences the severity of the *sn* phenotype. The most stringent requirement for singed protein appears to be in nurse cells, which have only minutes to complete a massive actin assembly that may require immediate bundling to prevent actin filament depolymerization. In the developing bristle there may be adequate time for other actin binding proteins to stabilize filaments and allow minimal organization and elongation. Both nurse cell cytoplasm transport and bristle extension provide excellent *in vivo* models for the functional analysis of actin binding proteins.

Chapter 4

CHARACTERIZATION OF BACTERIALLY-EXPRESSED SINGED

INTRODUCTION

The cloning of the *sn* gene revealed an open reading frame of 1536 nucleotides encoding a 57 kD protein (Paterson and O'Hare, 1991). Recently, the gene encoding echinoderm fascin was cloned and found to have 35% peptide identity and 67% peptide similarity with the *sn* gene (Bryan et al., 1993). Fascin was originally described as a 58 kD component of actin gels made from sea urchin egg extracts (Kane, 1976). When purified, fascin was found to have actin bundling activity. Actin bundles containing fascin are hexagonally packed, have a characteristic cross-banding pattern of about 12 nm periodicity, and contain a molar ratio of fascin to actin of 1:4.3 (Bryan and Kane, 1978; Cant et al., 1994; DeRosier and Censullo, 1981; DeRosier et al., 1977; Kane, 1976; Spudich and Amos, 1979). Fascin was localized to actin filament bundles in both sea urchin egg microvilli and coelomocyte filopodial extensions (Otto and Bryan, 1980; Otto et al., 1979; Otto et al., 1980). In addition to sequence homology between the *sn* and the *fascin* genes, singed also is required for the formation of actin filament bundles in nurse cells and developing bristles (see Chapter 3). I sought to determine whether the biochemical characteristics of singed resemble those of fascin. In this chapter, I describe the actin bundling properties of bacterially-expressed singed protein. The results suggest that *Drosophila* singed protein is functionally homologous to echinoderm fascin.

RESULTS

Low speed cosedimentation assay

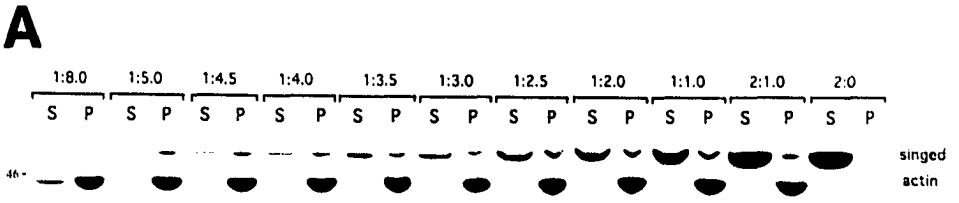
I used purified, bacterially-expressed singed protein to explore whether singed has functional homology with the actin filament bundling protein fascin. The singed protein used in my *in vitro* experiments was made as a recombinant protein in the pET expression system (Novagen) and contained three extra amino acids, glycine, serine, and histidine, at the amino terminus. The protein was approximately 57 kD (Figure 12A) and was recognized by singed monoclonal antibodies on western immuno-blots (not shown).

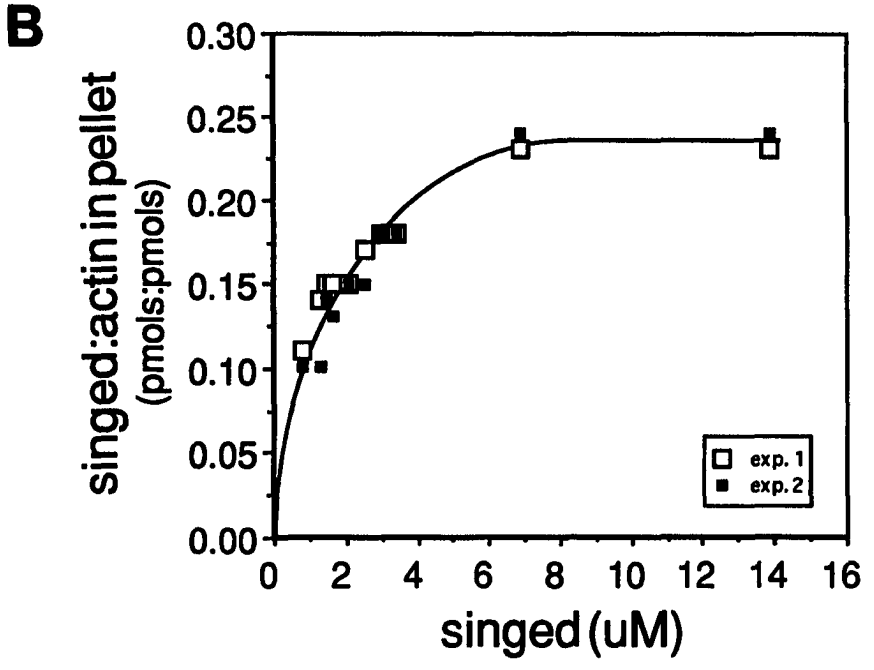
In preliminary studies, purified singed protein (3.5 μM) was mixed with F-actin (7 μM) in a 1:2 molar ratio and bundles of F-actin were visible by dark field microscopy (not shown). To quantitate the interaction between singed and actin, low speed cosedimentation assays were conducted on samples containing 7 μM phalloidin-stabilized F-actin and increasing concentrations of purified singed (0.87 μM - 14 μM) such that the singed:actin molar ratios ranged from 1:8 to 2:1 (Figure 12A). Samples were centrifuged at low speed to pellet actin filament bundles. The amount of protein in the soluble and pellet fractions was determined using scanning densitometry. Less than 2% of either F-actin or singed protein alone sedimented upon low speed centrifugation (Figure 12A, singed to actin ratio 2:0). To quantitate the stoichiometry of singed binding to actin, I measured the ratio of singed to actin in the pellet fractions (Figure 12B). The saturation of actin bundles with singed was reached when the amount of singed in the pellet no longer increased despite increasing singed protein concentration (compare 7 μM to 14 μM singed in Figure 12B). The molar ratio of singed to actin in saturated singed-actin bundles was calculated as approximately 0.23 from densitometry data. This can also be seen by extrapolating the saturation curve to the

Figure 12. Low speed cosedimentation of singed and actin

A. Mixtures of singed and F-actin at molar ratios of singed:actin varying from 1:8 to 2:1 were centrifuged at low speed. The distribution of actin and singed in the supernatant (S) and pellet (P) fractions were visualized by coomassie staining of SDS-PAGE gels. The concentration of F-actin remained constant at 7 μM, while the singed concentration increased from 0.87 μM to 14 μM.

B. Quantitation of two low speed co-sedimentation experiments was done using scanning densitometry. Exp 1 refers to quantitation of the protein bands shown in A; exp 2 refers to the same experiment repeated. The molar ratio of singed:actin in the pellet fraction is plotted against the total concentration of singed prior to centrifugation. The molar ratio of singed:actin in the pellet becomes saturated at a ratio of about 0.23, or 1:4.3.





ordinate in Figure 12B. The ratio of 0.23 converts to a singed:actin molar ratio of 1:4.3 and this is consistent with the stoichiometry of fascin-actin bundles of 1:4.6.

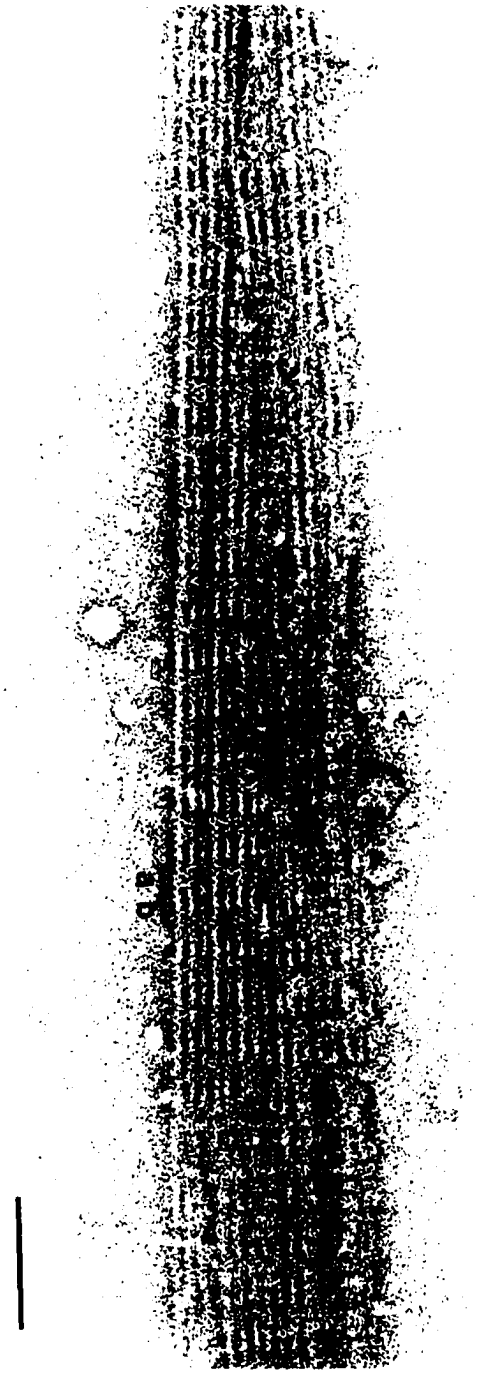
While both singed-actin and fascin-actin bundles contained a molar ratio of approximately 1:4.5, the conditions for saturation varied dramatically. Saturation of singed-actin bundles required a molar ratio of 1:1 singed:actin because most singed remained unbound. Saturation of fascin-actin bundles required a molar ratio of about 1:4 fascin:actin, in which most fascin was bound (Bryan and Kane, 1978). The high concentration of singed required to saturate actin suggested that singed bound actin with low affinity. To eliminate the alternative possibility that the singed protein preparation contained a mixture of active and inactive protein, I tested the bundling activity of both singed recovered from bundles and singed remaining soluble. Both these fractions retained qualitatively similar bundling activities compared to the original singed protein preparation (Chapter 2). The soluble fraction of singed retained bundling activity. Singed recovered from bundles maintained its ability to bundle F-actin, yet, as with the original singed preparation, most of the singed remained soluble. These data suggested that the singed protein preparation contained homogeneously active protein with a low affinity for actin. Bacterially-expressed sea urchin fascin is not soluble (Bryan et al., 1993) and therefore no direct comparison can be made with bacterially-expressed singed.

Ultrastructure of a singed-actin bundle

Electron microscopy of negatively stained actin bundles typically reveals a transverse cross-banding pattern characteristic of the bundling protein cross-linking actin filaments. Incubation of singed and phalloidin-stabilized F-actin at a 1:2 singed:actin ratio resulted in F-actin bundles with a

Figure 13. Electron micrograph of a negatively stained singed-actin bundle

Singed-actin filament bundles were formed by mixing purified bacterially-expressed singed protein and phalloidin-stabilized F-actin. Negative staining reveals transverse cross-bands appearing as doublets that represent singed protein cross-linking actin filaments. The small interval (bracket "a") within a single doublet is 12 nm ± 1.2, while the large interval between doublets (bracket "b") is 24.9 nm ± 1.7. A distinct characteristic view showing a continuous 12 nm transverse periodicity is suggested near the right end of this bundle. Magnification is 168,000. Scale bar represents 100 nm.



transverse cross-banding pattern (Figure 13). I saw two characteristic views of negatively stained singed-actin bundles. The bundle in Figure 13 had several regions of cross-band doublets. The interval between pairs of cross-bands within a doublet was 12 nm ± 1.1 (average of 15 intervals such as the bracket labeled "a" in bundle shown in Figure 13), whereas the interval between doublets was 24.9 nm ± 1.7 (average of 12 intervals such as the bracket labeled "b" in Figure 13). The larger space occurred at sites of actin filament crossover and thus the cross-band at this point is difficult to see. While the doublet view was more common, a region of continuous 12 nm cross-banding repeats and less discernible actin filaments is suggested near the right end of the bundle in Figure 13, and was clearly visible on other bundles. I measured the intervals between 8 continuous cross-bands on a different bundle and found a 12 nm transverse periodicity. Negatively stained fascin-actin bundles also have two similar characteristic patterns on electron micrographs depending on the orientation of the bundle in the microscope. The 12 nm transverse periodicity and the stoichiometry of singed:actin of 1:4.3 determined by cosedimentation assay closely resemble fascin:actin interactions. I could not determine whether singed-actin bundles contained uniformly polarized actin filaments.

DISCUSSION

Singed is functionally homologous to fascin

Several lines of evidence indicate that the *Drosophila sn* gene encodes a homolog of echinoderm fascin. *Drosophila sn* and sea urchin *fascin* encode proteins of similar molecular weight with 35% peptide identity and 67% overall similarity (Bryan et al., 1993). I have shown that actin filaments bundled with bacterially-expressed singed protein resemble fascin-bundled

actin filaments. Electron micrographs of negatively stained singed-bundled actin filaments and fascin-bundled actin filaments (Bryan and Kane, 1978; Kane, 1976; Spudich and Amos, 1979) both show parallel actin filaments with a transverse cross-banding pattern of about 12 nm. Analysis of fascin-actin bundles using optical diffraction and image reconstruction showed that the bundles consist of actin filaments arranged in a hexagonal lattice with 9 fascin cross-band links per 41 actin monomers (DeRosier and Censullo, 1981; DeRosier et al., 1977). Therefore, this model places restriction on the molar ratio of fascin to actin. The transverse periodicity and the stoichiometry of both fascin-actin bundles (12 nm and 1:4.6) and singed-actin bundles (12 nm and 1:4.3) agrees with the stoichiometry fixed by the geometry of hexagonally packed bundles described for fascin-actin bundles.

Chapter 5

IDENTIFICATION OF CRITICAL AMINO ACIDS IN FASCIN

INTRODUCTION

Fascin has been implicated in the organization of actin filaments bundles in many organisms. When sea urchin fascin was cloned (Bryan et al., 1993), the peptide sequence was found to have homology to the *Drosophila singed* (*sn*) gene product (Paterson and O'Hare, 1991) and HeLa cell p55 (Yamashiro-Matsumura and Matsumura, 1986; Yamashiro-Matsumura and Matsumura, 1985). Recently, mouse (Edwards et al., 1995), *Xenopus* (Holthius et al., 1994), and human (Duh et al., 1994; Mosialos et al., 1994) homologs of fascin have been described. Using the algorithm, Pileup (University of Wisconsin Genetic Computer Group, version 8.1, 1995), fascin homologs are about 35% identical and 60% similar, but amino acid conservation among fascin homologs does not reveal specific functional domain organization.

Fascin should contain two actin binding domains since the protein cross-links actin filaments as a monomer (Bryan and Kane, 1978; DeRosier and Censullo, 1981). Traditional biochemical approaches for mapping actin binding domains have had limited success. Truncated proteins expressed in *E. coli* have either been insoluble or incorrectly folded (R. Edwards, personal communication). A human 27 kD C-terminal fragment (Ono et al., 1994) and a mouse 30 kD C-terminal fragment (Edwards and Bryan, 1995) were generated using limited proteolysis and analyzed. The C-terminal half of human or mouse fascin appears to be able to bind, but not bundle, actin filaments and therefore contains at least one actin binding domain.

Genetic analysis can provide an *in vivo* complement to biochemical and structural approaches used to dissect the interaction between actin

binding proteins and actin. In yeast, elegant genetic analysis involving a combination of mutagenesis and suppressor screens defined the critical amino acids in both fimbrin and actin that are required for the fimbrin-actin interaction. Mutations in fimbrin (SAC6) can suppress mutations in actin (ACT1) and mutations in actin (ACT1) can suppress mutations in fimbrin (SAC6) (Adams and Botstein, 1989; Adams et al., 1989; Adams et al., 1991). This genetic evidence for physical interaction between fimbrin and actin was confirmed by actin bundling assays *in vitro* and protein colocalization *in vivo* (Drubin et al., 1988). Molecular analysis of the mutation sites revealed the specific amino acids involved in this interaction and these findings were consistent with structural models (Honts et al., 1994).

Drosophila is an ideal organism for mutagenesis and genetic analysis. The *Drosophila* fascin homolog, singed protein, has been well characterized in Chapters 3 and 4. Over 100 *sn* alleles have been described; however, few are candidates for mutations in the coding region. Molecular analysis of a 5 spontaneous *sn* alleles identified mutations in the promoter or 5' untranslated regions of the gene (Lindsley and Zimm, 1992). Many *sn* alleles are caused by transposable elements that typically insert into the promoter region, reducing *sn* transcription. EMS mutagenesis screens for female sterile mutations on the X chromosome yielded 8 EMS *sn* alleles (Gans et al., 1975; Komitopoulou, 1983; Mohler, 1977). Unfortunately, only 2 of these alleles are extant, M3 (Mohler, 1977) and 1421 (Komitopoulou, 1983). I conducted a large EMS mutagenesis screen to isolate additional EMS *sn* alleles. By analyzing EMS alleles of *sn*, I identified single amino acid changes that alter fascin function *in vivo*. I then used one of these new *sn* alleles in a second mutagenesis screen to identify a dominant suppressor of *sn* sterility.

RESULTS

EMS mutagenesis for new *sn* alleles

I performed an EMS mutagenesis screen to isolate new *sn* alleles with missense or nonsense mutations in the protein coding region. The mutagenesis screen was designed to identify genes affecting bristle morphology on the X chromosome (Figure 14). Flies were fed 20 mM EMS, a dose that is 5 mM less than is typically used, in order to reduce male sterility and lethality. I screened approximately 68,500 male progeny of mutagenized X-chromosomes and obtained 48 new bristle mutants on the X chromosome. Complementation analysis with *sn* and *forked*, two X-linked genes affecting bristle morphology, classified our 48 new bristle mutants as 15 *sn* alleles, 23 *forked* alleles, and 10 mutants that complemented *sn* and *forked* (Figure 14).

One *sn* allele had an intermediate phenotype; the bristles were moderately bent and the females were fertile. The remaining 14 *sn* alleles had severe phenotypes that resembled the null allele phenotype of sn^{X2}; they had gnarled bristles and the females were sterile. Complementation testing among the 10 non-*sn*, non-*forked* mutants placed them into two groups, A and B. Complementation group A contained 8 alleles that differed in severity and exhibited a variety of phenotypes including short, gnarled bristles (Figure 15 D, E), rough eyes, and poor viability with increased pupal lethality. I mapped the mutation in complementation group A to 1.38.5 using meiotic recombination. *furrowed* maps to 1.36.85 (Lindsley and Zimm, 1992) and *furrowed* mutants have a similar spectrum of phenotypes. Complementation testing revealed that mutants in complementation group A were *furrowed* alleles. Complementation group B contained two alleles that both exhibited a subtle thin, bent bristle phenotype and one allele had reduced female fertility. I have not determined a previously identified locus

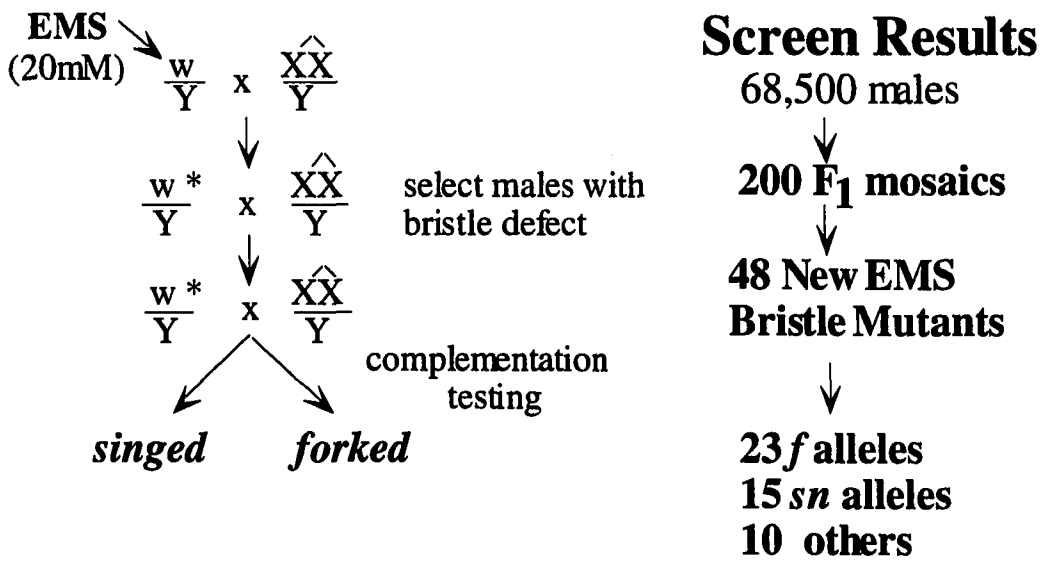
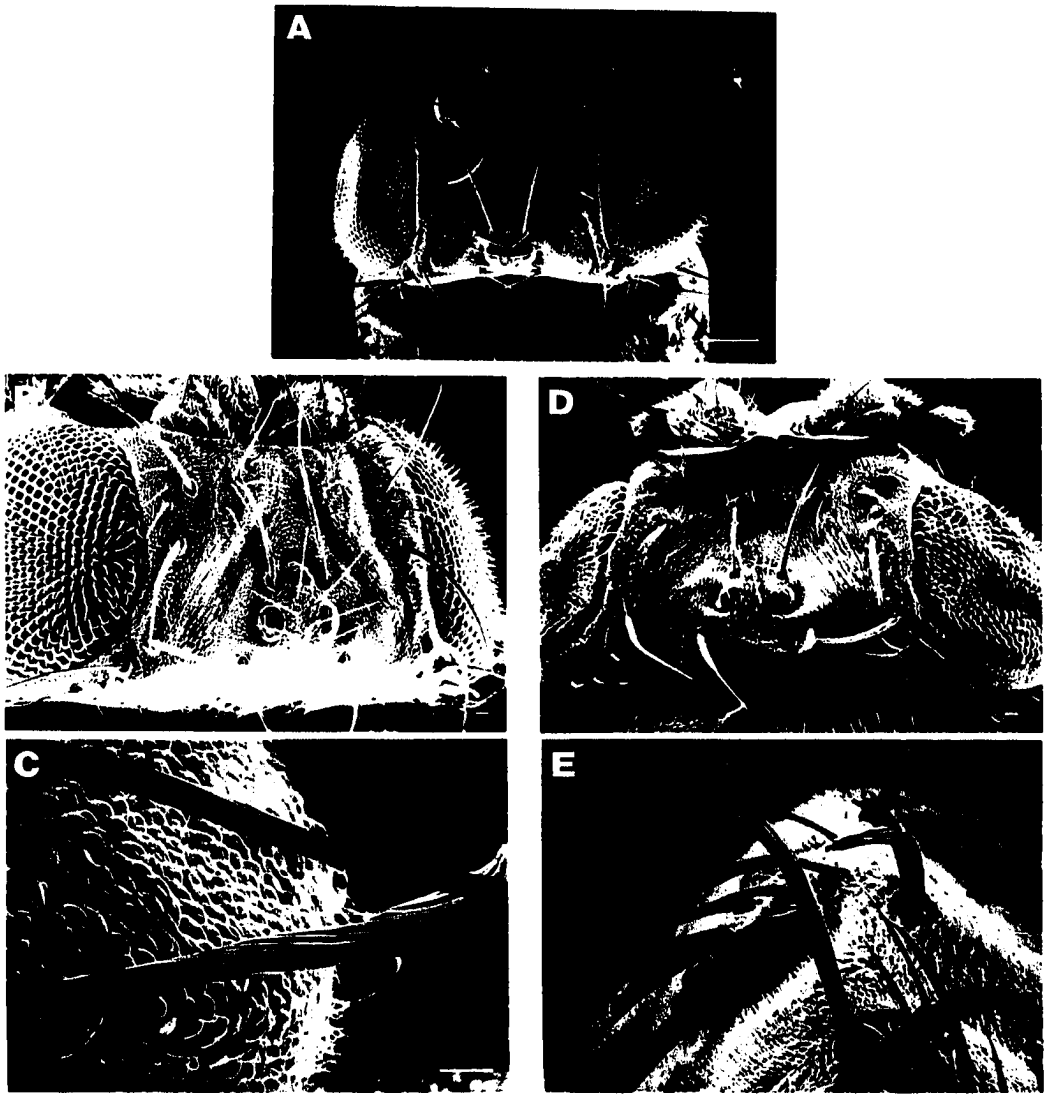


Figure 14. EMS mutagenesis screen for new *singed* (*sn*) alleles

Isogenic w^{1118} males were mutagenized and mated to *C(1)yf/Y* females (X^X represents compound X chromosomes). Male progeny with bristle defects were selected and independent lines were generated from those males that could stably transmit the bristle defect to their progeny. I tested new bristle mutants for complementation with *sn* and *forked,* two common bristle morphology mutants on the X chromosome.

Figure 15. Bristle phenotype of EMS mutants that complement *sn* and *forked*

SEM of adult head bristles on wild type (A), mutant *83* from complementation group B (B, C), and *furrowed*^{130} allele from complementation group A (D, E). The bristle phenotype on *83* is very subtle and variable. Some bristles are bent and many bristles are twisted (C). The bristle phenotype of *fw*^{130} is variable; many bristles appear as though they are broken and they have jagged tips (E). Scale bar in A represents 100 μm. Scale bars in B-D represent 10 μm.



affected in complementation group B.

Analysis of EMS *sn* alleles

We were primarily interested in analyzing those *sn* alleles in which the observed phenotype could be explained by a defect in protein function rather than an absence of protein. Therefore, I performed Northern and Western analysis on the 15 new EMS alleles and 2 previously described EMS *sn* alleles, M3 (Mohler, 1977) and 1421 (Komitopoulou et al., 1983). *sn* encodes 3 transcripts of 3.6-, 3.3-, and 3.0-kb (Paterson and O'Hare, 1991). These transcripts differ only in their polyadenylation site and the 3.3- and 3.0-kb transcripts are unique to ovaries. Northern analysis revealed that only 4 alleles, *G409E, 116, 121,* and S289N, contained significant levels of *sn* transcripts (Figure 16A). The ovary-specific transcripts were missing from sn^{116}. Western analysis of ovary extracts from these EMS *sn* alleles revealed that only two *sn* alleles, sn^{G409E} (fertile allele) and sn^{S289N} (sterile allele), contained detectable singed protein (Figure 16B).

Egg chambers from sn^{G409E} and sn^{S289N} were further analyzed using immunofluorescence with antibodies to *Drosophila* singed. Egg chambers from wild type flies contained fascin in border cells, posterior follicle cells, centripetal follicle cells and in the nurse cell cytoplasm (Figure 17A, (Cant et al., 1994). Egg chambers from both sn^{G409E} and sn^{S289N} contained fascin in a pattern consistent with wild type (Figure 17B, C).

The rapid phase of nurse cell cytoplasm transport is an actin dependent process. In egg chambers from wild type flies, a subcortical actin network is detected at the nurse cell membrane. Just prior to nurse cell rapid cytoplasm transport, cytoplasmic actin bundles form and extend from the plasma membrane to the nuclear membrane (Figure 17D). As the nurse cell cytoplasm is transported into the oocyte, the oocyte grows, and the nurse cells

Figure 16. Northern (A) and Western (B) analysis of EMS *sn* alleles

(A.) Female RNA was probed for *sn* and *rpA1* (Qian et al., 1987), as a loading control. Transcripts corresponding to the three *sn* transcripts of 3.6, 3.3, and 3.0 kb were detected in wild type. *sn* transcripts were detected in only four EMS alleles, sn^{G409E}, sn^{116}, sn^{121}, and sn^{S289N}. The smaller, ovary-specific transcripts were absent from sn^{116}. sn^{1421} and sn^{M3} were generated in previous EMS mutagenesis screens (Komitopoulou et al., 1983; Mohler, 1977)respectively). 2.2- and 4.4-kb molecular size markers are indicated on the left.

(B) Ovary protein extracts were immunoblotted with singed monoclonal antibody 7C for singed, and with kelch monoclonal antibody 1B as a loading control. Fascin was detected only in sn^{G409E} and sn^{S289N}. 47- and 69 kD molecular weight markers are indicated on the left.

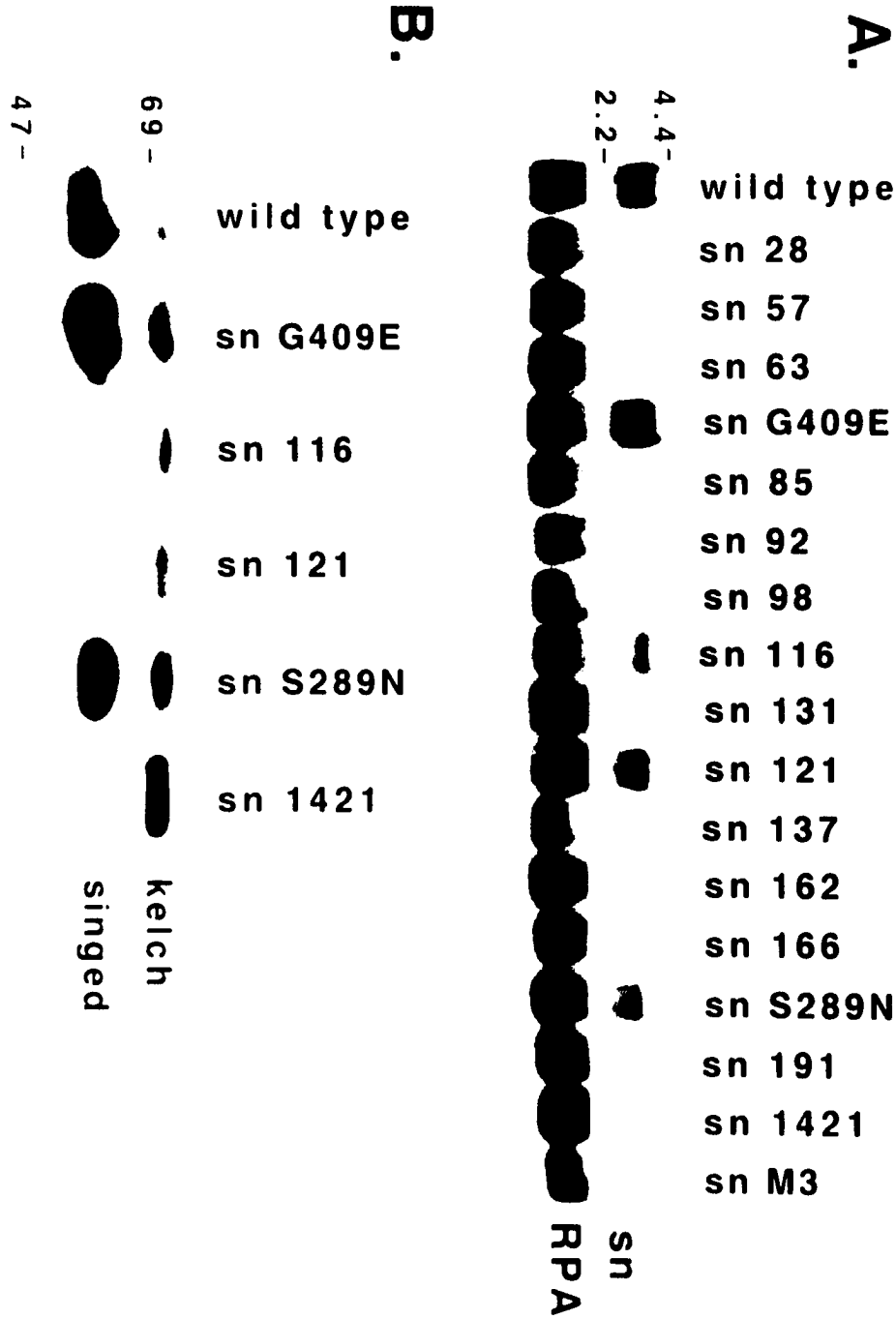


Figure 17. Egg chamber phenotypes of sn^{G409E} and sn^{S289N}

Egg chambers from wild type (A, D, G), the fertile allele sn^{G409E} (B, E, H) and the sterile allele sn^{S289N} (C, F, I) were stained either with singed monoclonal antibody (A, B, C) to detect fascin or rhodamine phalloidin (Rh Ph, D-I) to detect filamentous actin. Fascin is highly expressed in the nurse cell cytoplasm, border cells (BC), centripetal follicle cells (CFC), and posterior follicle cells (PFC) in wild type (A), sn^{G409E} (B), and sn^{S289N} (C). Stage 10B egg chambers from wild type females (D) have a subcortical actin network just beneath the cell membrane and an extensive cytoplasmic actin bundle network (CAB). In wild type, all of the nurse cell cytoplasm is transported into the oocyte and the nurse cells regress (G). Although fascin is expressed in sn^{G409E} (B), the cytoplasmic actin bundles appear sparse and disorganized (E). At stage 14, a sn^{G409E} egg (H) shows that not all of the nurse cell cytoplasm is transported into the oocyte and the egg is only about 75% the size of a wild type stage 14 egg (G). Egg chambers from sn^{S289N} females resemble those seen in null *singed* alleles (Cant et al., 1994); cytoplasmic actin bundles are nearly absent (F), the rapid phase of cytoplasm transport is disrupted, and mature eggs (I) are only half the size of wild type eggs (G).

regress (Figure 17G). sn^{G409E} was a fertile allele, but the cytoplasmic actin bundles appeared faint and disorganized (Figure 17E). Not all of the nurse cell cytoplasm was transported into the oocyte and the eggs were about 75% the size of wild type eggs (Figure 17H). While the actin defect in sn^{G409E} nurse cells was subtle, the actin defect in sn^{S289N} was severe and appeared similar to the defect seen in null sn^{X2} mutants (Cant et al., 1994); the subcortical actin was normal but the cytoplasmic actin bundles were nearly absent (Figure 17I) The rapid phase of cytoplasm transport in this allele was disrupted and the oocyte remained only half the size of a wild type egg (Figure 17I).

sn^{G409E} and sn^{S289N} have bristle phenotypes that correlate with the severity of the oogenesis phenotypes (Figure 18). sn^{G409E} has an intermediate bristle phenotype; the bristles have multiple bends but are not gnarled (Figure 18B,E). sn^{S289N} has a moderately gnarled bristle phenotype (Figure 18C, F); however, the bristles are not as severely gnarled as bristles on the null sn^{X2} mutant (Figure 18D).

The phenotypes of sn^{G409E} and sn^{S289N} suggested that the fascin expressed in these alleles was abnormal (Table 4). To identify the mutations, the *sn* open reading frame was amplified from genomic DNA isolated from the parental line (isogenic w^{1118}), sn^{G409E}, or sn^{S289N} and then sequenced. sn^{G409E} had a single nucleotide G-->A transition in codon 409 that changed a GGA (glycine) to GAA (glutamic acid) (Figure 19). sn^{S289N} had a single nucleotide G-->A transition in codon 289 that changed AGC (serine) to AAC (asparagine) (Figure 19).

The severe phenotypes of sn^{S289N} suggested that the mutation in serine 289 dramatically altered fascin function. In sn^{S289N} mutants, the bristle phenotype was only slightly less gnarled than the severely gnarled phenotype

Figure 18. Bristle phenotype of sn^{G409E} and sn^{S289N}

SEM of adult bristle on wild type (A), sn^{X2} null allele (D), sn^{G409E} (B, E), and sn^{S289N}(C, F). sn^{S289N} has a moderately severe bristle phenotype but they are not as severe as the gnarled bristles on sn^{X2}. sn^{G409E} has a subtle multiple bends bristle phenotype. Scale bars in A and D represent 100 μm. Scale bars in B, C, E, F represent 10 μm.

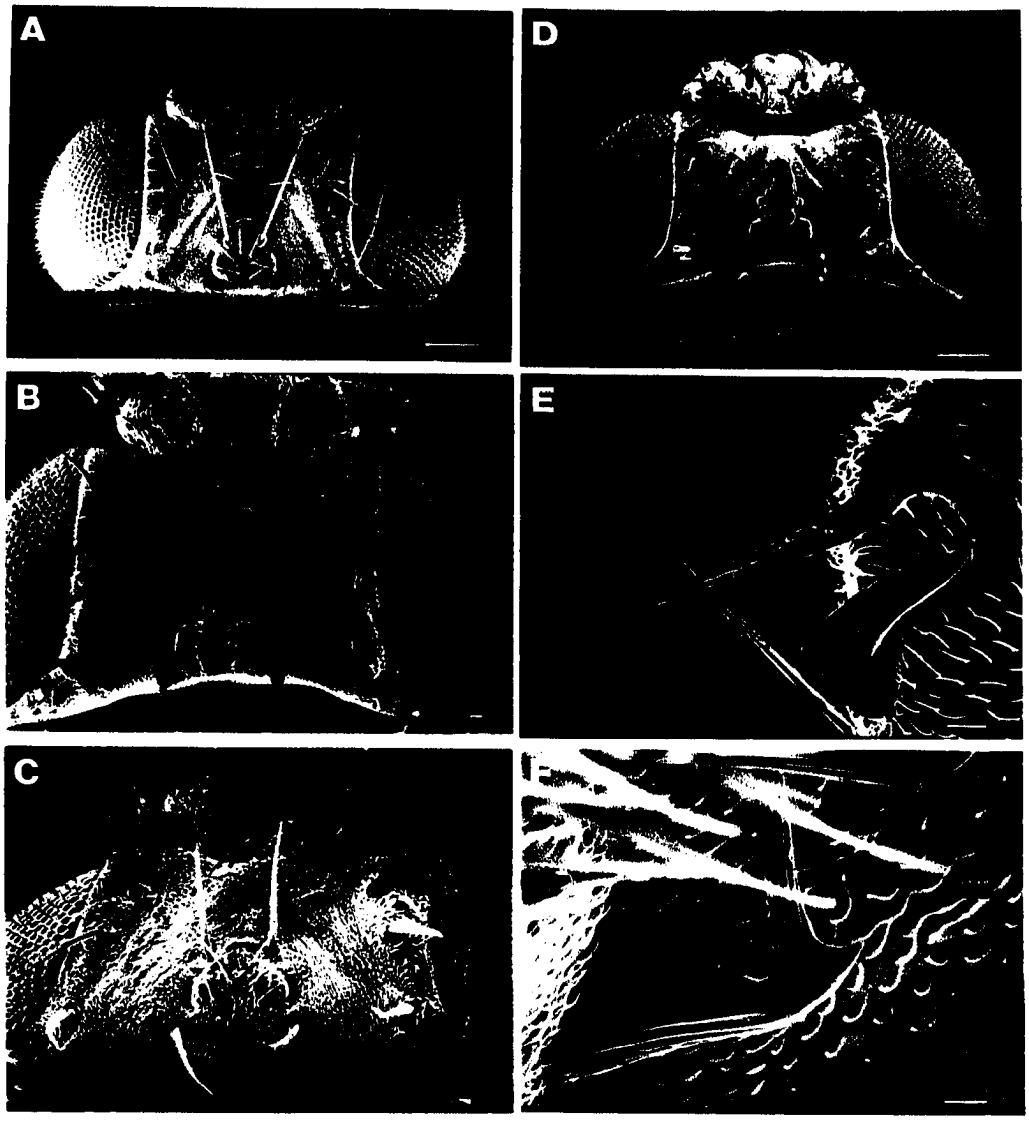


Figure 19. Best Fit alignment of *Drosophila* (FLY) and sea urchin (SU) fascin

The alignment of *Drosophila* and sea urchin fascin was generated with the Best Fit program in the Wisconsin Package of GCG (version 8.1, 1995). Numbers indicate amino acid number. Amino acids affected in sn^{G409E} and sn^{S289N} are indicated in outlined letters that are underlined. The sn^{G409E} mutation lies in a highly conserved domain of 21 amino acids that is underlined with asterisks. *Drosophila* and sea urchin fascin are 35% identical, but in this 21 amino acid domain they are 71% identical.

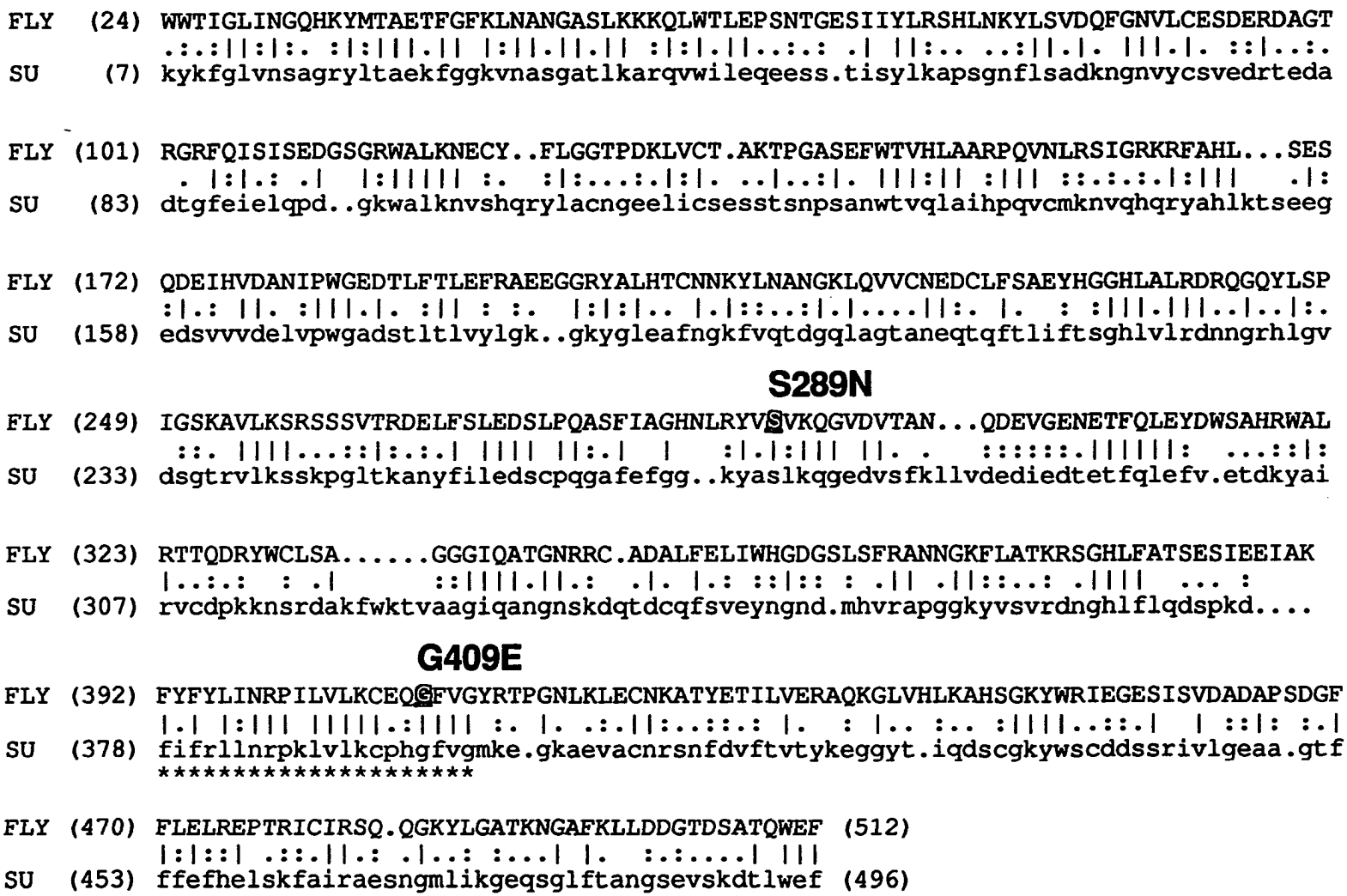


Table 4. EMS induced *singed* alleles

			PHENOTYPES		
Allele	**Mutation**	**Overall Severity**	**Bristle**	**Egg Size**	**Fertility**
sn^{G409E}	G409E	interm.	multiple bends	75%	fertile
sn^{S289N}	S289N	severe	mod. gnarled	50%	sterile
sn^{28}	unknown	null	severely gnarled	50%	sterile
$sn^{SupS289N}$	S251F, S289N	interm.	subtly bent	80%	fertile

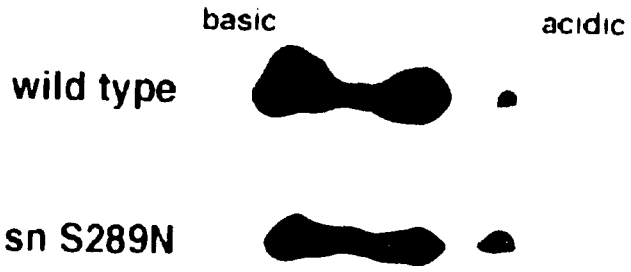
of the null allele sn^{X2} and the egg chamber phenotype mimicked that seen in null sn^{X2} allele. Serine 289 was contained in a region with no homology to actin binding domains identified in other proteins. Many isoforms of fascin have been identified by 2-D gel electrophoresis and Western blotting (Edwards et al., 1995; Yamashiro-Matsumura and Matsumura, 1985). I used 2D gel-electrophoresis and Western blotting to analyze ovary extracts from wild type and sn^{S289N} females. Many fascin isoforms were resolved; however, the isoforms present in wild type ovaries were also present in mutant sn^{S289N} ovaries (Figure 20). I also expressed mutant fascin^{S289N} protein in the same pET 14b bacterial expression system (Novagen) used to express soluble wild type protein (Cant et al., 1994). However, the mutant protein was not soluble and could not be purified (data not shown).

EMS mutagenesis for dominant suppressors of *singed*

sn^{S289N} was the best allele for further genetic analysis because it expressed a mutant protein and was sterile. To identify potential modifiers of fascin function, I performed a screen for dominant suppressors of *sn* sterility. Genetic screens for suppressors of female sterility are extremely sensitive because sn^{S289N} females will only be fertile if they carry mutations that can dominantly suppress the sn^{S289N} sterility. I mutagenized sn^{S289N} males with 25 mM EMS and then mated them to heterozygous sn^{S289N} females (Figure 21). 28,100 out of 38,100 sn^{S289N} females were screened at 25° C, and the remaining 10,000 were screened at 18° C to test for cold sensitive suppressors. Homozygous sn^{S289N} female progeny that contained mutagenized chromosomes were screened for fertility by placing 20 females and 10 sn^{S289N} males in vials. Any F2 progeny in these vials must have been derived from a female sn^{S289N} that carried a dominant suppressor. Progeny were subsequently tested for fertility. Although no progeny were ever

Figure 20. Fascin isoforms in wild type and sn^{S289N} ovary extracts

Ovary extracts from wild type and from sn^{S289N} were analyzed by 2D electrophoresis and immunoblotting with singed monoclonal antibody 7C. Many isoforms of fascin are detected; however the isoforms present in wild type are also present in sn^{S289N} ovaries. The isoform pattern is not affected by the S289N mutation.



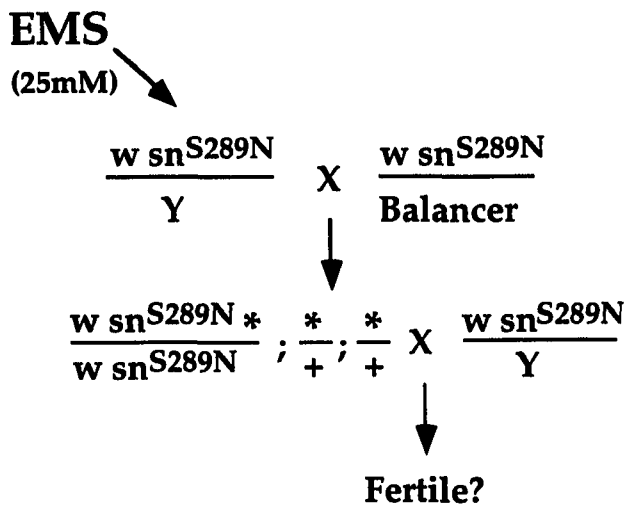


Figure 21. EMS mutagenesis screen for dominant suppressors of sn^{S289N} sterility

We mutagenized *w* sn^{S289N} males and mated them to heterozygous balanced sn^{S289N} female virgins. Homozygous sn^{S289N} female progeny containing mutagenized chromosomes were screened for fertility. Homozygous sn^{S289N} females should be sterile unless they carry a dominant suppressor of *singed* sterility. Any progeny obtained were further screened for fertility to determine if they inherited a suppressor.

obtained from unmutagenized sn^{S289N} females, about 5% of the vials containing sn^{S289N} females with mutagenized chromosomes produced 1-3 progeny. These progeny were never fertile. One suppressor, *Su(sn)* was recovered from the 18° C portion of the screen. sn^{S289N} females carrying one copy of the *Su(sn)* mutation were fertile at 18° C and at 25° C. *Su(sn)* not only restored sn^{S289N} fertility, but also suppressed the gnarled bristle morphology defect. sn^{S289N} females with one copy of *Su(sn)* had an intermediate multiple bends bristle phenotype and sn^{S289N} males with one copy of *Su(sn)* had a very weak bristle defect visible only as subtle bends at the tips of the bristles (data not shown).

We used the bristle suppression phenotype to map the *Su(sn)*. When *Su(sn)* males were mated with *C(1) yf/Y* females, all male progeny had the suppressed bristle phenotype indicating that the *Su(sn)* was on the X chromosome. In order to map the *Su(sn)* with respect to sn^{S289N}, I counted recombination events between *Su(sn)* and sn^{S289N} in heterozygous *Su(sn)*, sn^{S289N}/++ females. There were 0/7968 recombination events between *Su(sn)* and sn^{S289N}. Therefore, the *Su(sn)* mutation appeared to be within .038 ($p<0.05$) map units or 22.8 kb of sn (calculated as per Ashburner 1989, p. 458). Since *Su(sn)* was tightly linked to *singed* and also appeared to suppress both the gnarled bristle and egg chamber phenotypes of sn^{S289N}, I tested whether *Su(sn)* was an intragenic suppressor of sn^{S289N}. I sequenced the *sn* open reading frame of sn^{S289N}, *Su(sn)* males and found that in addition to the original S289N mutation in *sn*, there was an additional C-->T transition in codon 251 that changed a serine (TCC codon) to phenylalanine (TTC) (Figure 22). This second mutation is most likely a suppressor mutation although we cannot rule out the possibility that the mutation is a polymorphism and the suppressor is a different mutation in a gene that is

Figure 22. *sn*^{SupS289N} is an intragenic mutation in singed

sn^{SupS289N} contains both the original S289N mutation and a mutation that changes S251 to F (phenylalanine). While S289N appears to inactivate fascin, the additional mutation S251F appears to partially correct fascin function.

tightly linked to *singed*. Flies containing the original S289N mutation and the suppressor S251F mutation in the same *sn* open reading frame are referred to as $sn^{SupS289N}$.

Filamentous actin networks were examined in egg chambers from dominantly suppressed $sn^{SupS289N}/sn^{S289N}$ females and in homozygous $sn^{SupS289N}$ females using rhodamine phalloidin (Figure 23). Egg chambers from the dominantly suppressed $sn^{SupS289N}/sn^{S289N}$ females contained sparse, unorganized nurse cell actin bundles and the eggs were about 70% percent the size of wild type eggs (Figure 23B, E). Homozygous $sn^{SupS289N}$ females also had defects in nurse cell rapid cytoplasm transport. While nurse cell cytoplasmic actin bundles were present (Figure 23C), they appeared faint and less organized than in wild type and the eggs were about 80% the size of wild type eggs (Figure 23F). I consider $sn^{SupS289N}$ to be an intermediate allele of *sn* because it has a subtle defect in nurse cell cytoplasm transport and is fertile (Table 4).

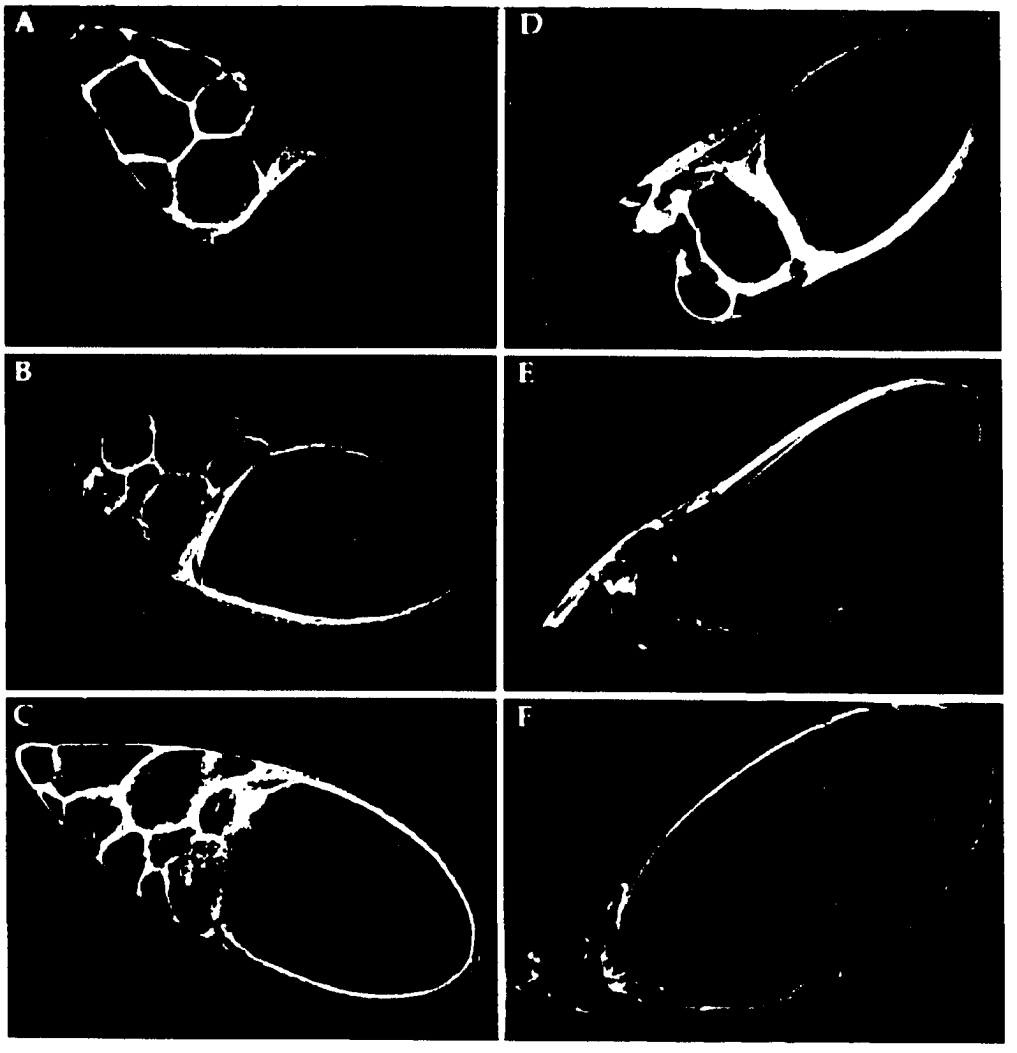
DISCUSSION

Glycine 409 and Serine 289 are important to fascin function

While fascin is known to bundle actin filaments as a monomer, neither the actin binding sites nor mechanisms of its regulation are known. I generated and sequenced EMS alleles of the *Drosophila singed* gene, which encodes fascin, to identify amino acids that are critical to fascin function *in vivo*. I identified two separate amino acid substitutions that each affect fascin function and result in *sn* phenotypes. Two lines of evidence indicate that changes in glycine 409 and serine 289 codons are the relevant mutations. These lesions both involve G-->A transitions, the most common mutation caused by EMS (Ashburner, 1989a), and no additional lesions were found in

Figure 23. Egg chamber phenotype of $sn^{SupS289N}$

The dominant suppressor of sn^{S289N} sterility screen identified an intragenic mutation in *singed* referred to as $sn^{SupS289N}$. $sn^{SupS289N}$ contains an additional mutation, S251F, in the *singed* gene that can restore function to fascin^{S289N}. sn^{S289N} females are sterile; nurse cell cytoplasmic actin bundles are nearly absent (A), the rapid phase of cytoplasm transport is disrupted, and a mature egg from sn^{S289N} is only half the size of a wild type egg (D). Dominantly suppressed $sn^{SupS289N}/sn^{S289N}$ females are fertile and have sparse, disorganized cytoplasmic actin bundles (B). These few cytoplasmic bundles allow mature eggs to grow to about 70% the size of wild type eggs (D). Homozygous $sn^{SupS289N}/sn^{SupS289N}$ females are fertile and have even more cytoplasmic actin bundles (C), although the bundles are less abundant and more disorganized than wild type (Figure 17D). The increase in cytoplasmic actin bundles appears to allow mature eggs to grow to about 80% the size of wild type (F).



the coding region. Secondly, the amino acids affected are conserved in other fascin homologs and the mutations lead to non-conservative amino acid substitutions.

The G409E mutation may highlight a critical amino acid in an important functional domain. However, since sn^{G409E} is an intermediate *sn* allele, fascin function must be only partially affected. Glycine 409 is conserved in all species analyzed to date (Bryan et al., 1993; Duh et al., 1994; Edwards et al., 1995; Holthius et al., 1994; Mosialos et al., 1994; Paterson and O'Hare, 1991; Yamashiro-Matsumura and Matsumura, 1985) and lies in the C-terminal half of the protein in a region of 21 amino acids that is highly conserved. In this region, *Drosophila* fascin is 71% identical to sea urchin fascin, 57% identical to human and *Xenopus* fascins, and 50% identical to mouse fascin. One actin binding site is contained within the C-terminal half of fascin (Edwards and Bryan, 1995; Ono et al., 1994). It will be interesting to determine whether this actin binding site maps to the conserved domain of 21 amino acids. Alternatively, the conserved domain is hydrophobic and the introduction of a negatively charged glutamic acid may disrupt the tertiary structure of a C-terminal domain that interacts with actin.

The phenotype of sn^{S289N} is similar to that seen in the protein null alleles, sn^{X2} (Cant et al., 1994) and sn^{28} (Table 4). All are female sterile due to an absence of nurse cell cytoplasmic actin bundles. Bristles on sn^{S289N} flies are gnarled, although not as severely as those on protein null mutants. This single amino acid substitution in the middle of the protein appears to nearly inactivate fascin. Serine 289 is conserved in human, mouse, and sea urchin fascins, but in *Xenopus* the corresponding amino acid is leucine.

The S289N mutation could affect protein tertiary structure. When $fascin^{S289N}$ is expressed in the same *E. coli* expression system used to express

wild type *Drosophila* fascin, the mutant protein is insoluble. This suggests that the S289N mutation significantly alters the physical properties of fascin. Fascin^{S289N} solubility could be affected *in vivo* as well; however, the protein localization in mutant egg chambers is similar to that seen in wild type. Another possibility is that the S289N mutation could alter actin binding domains of the protein by affecting fascin's tertiary structure. Chou-Fasman and Garnier-Osguthorpe-Robson structure predictions (Peptide structure, GCG) for *Drosophila* fascin both indicate that the S289 lies in a 15 amino acid beta sheet. Since an actin binding site has been mapped to the C-terminal half of human and mouse fascins (Edwards and Bryan, 1995; Ono et al., 1994), the S289N mutation may disrupt the alignment between N-terminal and C-terminal actin binding domains. The consequences of this mutation will be more easily understood once the three dimensional structure of fascin is determined.

Many fascin isoforms have been described by two dimensional gel electrophoresis. In humans, acidic fascin isoforms may be generated by phosphorylation. Purified HeLa cell fascin can be phosphorylated *in vitro* with protein kinase C, and *in vivo* by metabolic labeling (F. Matsumura, personal communication). 2D gel analysis of *in vitro* and *in vivo* phosphorylated human fascin reveals that $^{32}PO_4$ is not incorporated into the abundant basic isoforms but rather it is incorporated into the minor acidic isoforms that are visible primarily by autoradiography. Since I evaluated only the abundant major isoforms in wild type and *sn*^{S289N}, I cannot predict whether the serine 289 to asparagine mutation interferes with phosphorylation.

In mouse, the major basic fascin isoforms do not appear to result from phosphorylation. Standard 2D gel electrophoresis and acid-urea gel

electrophoresis of recombinant fascin resolve similar isoforms (Edwards et al., 1995), suggesting that phosphorylation is not the cause of the different major isoforms. No isoforms were absent in the ovaries from sn^{S289N} females. Therefore, the post-translational modifications of fascin that generate these basic isoforms are not affected by the mutation.

Few EMS *sn* alleles express protein

Surprisingly, sn^{S289N} and sn^{G409E} were the only two *sn* alleles out of 17 examined that expressed protein. EMS is known to cause point mutations and occasionally small deletions or duplications (Ashburner, 1989a) and EMS alleles often contain mutations in the protein coding region. All 70 EMS alleles of *nina* a, the *Drosophila* homolog of cyclophillin A (Ondek et al., 1992), and 24 alleles of *torpedo*, the *Drosophila* EGF receptor (Clifford and Schüpbach, 1994), contained mutations in the coding region. The presence of EMS induced mutations in the protein coding region is not restricted to receptor molecules. A high frequency of EMS mutations in the open reading frame have been found in the *rough* homeobox gene (Heberlein et al., 1994) and the gene encoding the *snake* protease (Smith et al., 1994).

While the literature suggests that the vast majority of EMS alleles contain small mutations in the coding region, I find that only 4 out of 17 alleles made stable mRNA, and only 2 of these 4 expressed protein. The 2 null alleles that expressed RNA but not protein can easily be explained by degradation of unstable proteins. But the predominance of null alleles that expressed neither RNA nor protein is difficult to explain. Mutations in these alleles may either disrupt the *sn* promoter or result in the production of an unstable transcript. The chromatin structure of the *sn* promoter region may allow this region to be especially susceptible to mutation by EMS.

sn alleles expressing protein appear to be difficult to obtain. Mutant forms of singed protein may not be tolerated, possibly resulting in dominant lethality. For example, a mutation disrupting an actin binding domain may allow the mutant protein to bind individual actin filaments and prevent actin bundle formation. Fascin is expressed in many cells during embryogenesis and generalized disruption of actin bundle formation would likely impair many developmental processes and result in lethality. Therefore, predominantly null alleles were recovered from our screen.

Alternatively, many amino acid substitutions in *sn* may not result in visible phenotypes. In the course of our screen, EMS mutations in the coding region may have been induced that did not alter protein function enough to disrupt bristle extension. However, evolutionary constraints on protein structure and function should limit the ability to induce invisible mutations. The mutation frequency in *sn* was similar to the mutation frequency in *forked* and *furrowed* suggesting that most mutations in *sn* were visible.

Another possible explanation for the difficulty in obtaining *sn* alleles expressing protein is redundancy. Small alterations in fascin function may not lead to defective bristle morphology if the cell contains additional actin cross-linking proteins that are partially redundant to fascin. In bristles of the null allele $singed^{X2}$, some actin bundles are present, although they are thin and very disorganized (Cant et al., 1994; Tilney et al., 1995). If bristle cells contain other actin cross-linking proteins that are partially redundant with fascin, then our screen would select only those mutations in fascin that were not compensated for by other actin cross-linking proteins. The screen would then be biased toward selection of *sn* alleles with severe defects in protein function or with no protein expression.

One candidate for an additional actin cross-linking protein in bristle development is forked. While the *forked* sequence does not reveal any homology to any known actin cross-linking protein, the protein is localized to the actin bundles in developing bristles and *forked* mutants have very small actin bundles (Petersen et al., 1994; Tilney et al., 1995). If forked and fascin have partially redundant actin cross-linking activity, then overexpression of forked may suppress *singed* bristle phenotypes. I used *forked* transformants (Petersen et al., 1994) to overexpress forked protein in intermediate (sn^{G409E}) and severe (sn^{S289N}) alleles but no suppression of the *sn* bristle phenotype was observed.

Characterization of an intragenic suppressor of sn^{S289N}

Since sn^{S289N} mutants express a mutant form of fascin, I chose this allele to use in a screen for dominant suppressors that would restore fertility to mutant females. Suppression can be achieved by many mechanisms. Compensatory mutations in actin could be identified if the S289N mutation affected an actin binding domain of fascin. Mutations in other proteins that interact with fascin or that by-pass the fascin requirement could be identified. Finally, if S289N alters the tertiary structure of fascin, then mutating an additional amino acid elsewhere in the protein may correct the protein's tertiary structure. I carried out a fairly large suppressor screen, but only one suppressor was identified. This could imply that fascin does not interact with many proteins other than actin. The absence of suppressor mutations in actin may indicate that S289N does not lie in a discrete actin binding site, that compensatory mutations in actin may be lethal, or that suppression would require compensatory mutations in both non-muscle actin isoforms. Finally, the structure of fascin^{S289N} may be so severely disrupted that subtle mutations in other proteins cannot suppress the defect.

The single suppressor I identified was an intragenic mutation in *sn*. While sn^{S289N} mutants have gnarled bristles and are sterile, the additional change of serine 251 to phenylalanine restored much of fascin's function. $sn^{SupS289N}$ females have moderately straight bristles that bend at the tips and they produce mature eggs that are about 80% the size of wild type eggs. While serine 289 is conserved in all fascin homologs (Bryan et al., 1993; Duh et al., 1994; Edwards et al., 1995; Mosialos et al., 1994; Paterson and O'Hare, 1991; Yamashiro-Matsumura and Matsumura, 1985) except *Xenopus* (Holthius et al., 1994), serine 251 is not conserved in other fascins. In sea urchin fascin this residue is a glycine, while in both human and *Xenopus* it is a proline. In *Drosophila* fascin, there are 10 serines between amino acid 246 and amino acid 289 that may be important for fascin protein folding. If the original serine 289 to asparagine mutation altered fascin's tertiary structure, the mutation serine 251 to phenylalanine may partially correct this structural defect. Both the S289N and S251F mutations draw attention to a central region of fascin.

Through genetic analysis I have generated three new *singed* alleles with mutations in the protein coding region and identified amino acids affected in each allele. I can now target these regions for more detailed biochemical characterization of fascin. Furthermore, these new *singed* alleles will be useful in future genetic analysis.

Chapter 6

GENETIC ANALYSIS OF *SINGED* AND *QUAIL*

INTRODUCTION

The formation of an extensive array of cytoplasmic actin filament bundles in *Drosophila* oogenesis is tightly regulated. In other systems, actin bundle assembly and stability is found to be a complex process that often requires at least two actin cross-linking proteins. A number of proteins such as α-actinin, fascin, fimbrin, and villin belong to the family of F-actin cross-linking proteins. It is intriguing that different F-actin crosslinking protein often co-exist in a single cell type, and often appear to be jointly required for the formation of certain actin bundle assemblies. Actin cross-linking proteins can be important in the organization, initiation, or stability of an actin bundle and, therefore, can affect different aspects of actin bundling (Matsudaira et al., 1983).

Actin bundles in the microvillar structure that constitutes the brush border microvilli of intestinal epithelia are organized by villin and fimbrin, two actin cross-linking proteins (for review (Louvard et al., 1992). Villin appears to be important for the initiation of actin bundle formation. When villin is expressed in fibroblast cells that do not normally express villin, it can induce actin reorganization and the formation of small microvillus-like projections from the cell surface (Franck et al., 1990; Friederich et al., 1989; Friederich et al., 1992). During initial gut development, villin is concentrated at the apical pole as the microvilli begin to form, (Shibayama et al., 1987). Fimbrin does not appear at the apical pole of enterocytes until two days after villin, and its expression coincides with an increased organization of the microvillus projections (Shibayama et al., 1987). Fimbrin, like fascin, is known to cross link actin filaments into highly organized, hexagonal arrays.

Fimbrin and villin show different sensitivities to calcium concentration. During microvillus formation *in vivo*, each protein may provide a unique function in addition to actin crosslinking activity. Villin could play a critical role in the initiation of actin filament bundle formation and brush border morphogenesis while fimbrin could be important in organizing and stabilizing the actin filament bundles.

In *Drosophila* egg chamber development, the formation of a specialized network of cytoplasmic actin bundles in nurse cells also depends upon at least two actin cross-linking proteins, the products of the *singed* (*sn*) and *quail* (*qua*) loci. In female sterile alleles of *sn* and *qua*, nurse cell cytoplasm transport into the developing oocyte is disrupted. *sn* and *qua* both encode proteins with homology to actin cross-linking proteins and both are required for cytoplasmic actin filament bundle formation (Bryan et al., 1993; Cant et al., 1994; Mahajan-Miklos and Cooley, 1994b; Paterson and O'Hare, 1991). Nurse cell cytoplasm transport provides an ideal system to study the role of these actin binding proteins *in vivo*.

As discussed in Chapter 4, singed is a functional homolog of the sea urchin actin bundling protein, fascin (Cant et al., 1994). Fascin is a well characterized monomeric actin bundling protein that cross-links actin filaments into highly organized, hexagonal arrays (Bryan and Kane, 1978; DeRosier and Censullo, 1981; DeRosier et al., 1977; Spudich and Amos, 1979). Fascin's actin cross-linking activity is not sensitive to calcium, magnesium, or ATP (Bryan and Kane, 1978).

The quail protein is also required for cytoplasmic actin bundle formation in *Drosophila* nurse cells. Quail co-localizes with the subcortical actin filaments prior to the formation of the cytoplasmic actin network and then co-localizes with the cytoplasmic actin bundle networks during rapid

cytoplasm transport (Mahajan-Miklos and Cooley, 1994b). Quail shares sequence homology with the actin cross-linking protein, villin (reviewed in Introduction, Mahajan-Miklos and Cooley, 1994). Quail homology to villin, its protein localization to actin bundles, and its requirement for cytoplasmic actin bundle assembly suggest that quail cross-links actin filaments in nurse cell cytoplasm (Mahajan-Miklos and Cooley, 1994b). Biochemical analysis of bacterially expressed quail protein confirmed its ability to bundle actin filaments with a stoichiometry of 1:3 quail:actin, consistent with villin's cross-linking activity (Mahajan-Miklos, 1995). It is not known if quail is calcium sensitive or if it has villin's capping, severing, or nucleating functions. In addition to its cross-linking activity, quail may support cytoplasmic actin bundle formation by promoting the nucleation of new actin filaments in the cytoplasm by capping and severing subcortical actin filaments (Mahajan-Miklos and Cooley, 1994b).

Both fascin and quail are required to organize the nurse cell cytoplasmic actin bundle network (Cant et al., 1994; Mahajan-Miklos and Cooley, 1994b). These proteins may act together and/or support distinct aspects of cytoplasmic actin bundle formation. I used genetic techniques to further analyze the function of fascin and quail in nurse cells. By examining the phenotypes of *sn* mutant egg chambers that were mutant for *qua* or overexpressed quail protein, I found that mutations in *qua* could enhance the *sn* oogenesis phenotypes. On the other hand, overexpression of *qua* could suppress the *sn* oogenesis phenotype.

RESULTS

Mutations in *quail* enhance the *singed*^{G409E} oogenesis phenotype

To determine if fascin and quail act together to form actin bundles I used genetic, second site non-complementation analysis. Both *sn* and *qua* are

fully recessive mutations. I used intermediate *sn* and *qua* alleles to simultaneously reduced the dose of wild type fascin function and quail function and examined the effect on actin bundle formation and nurse cell cytoplasm transport in egg chambers.

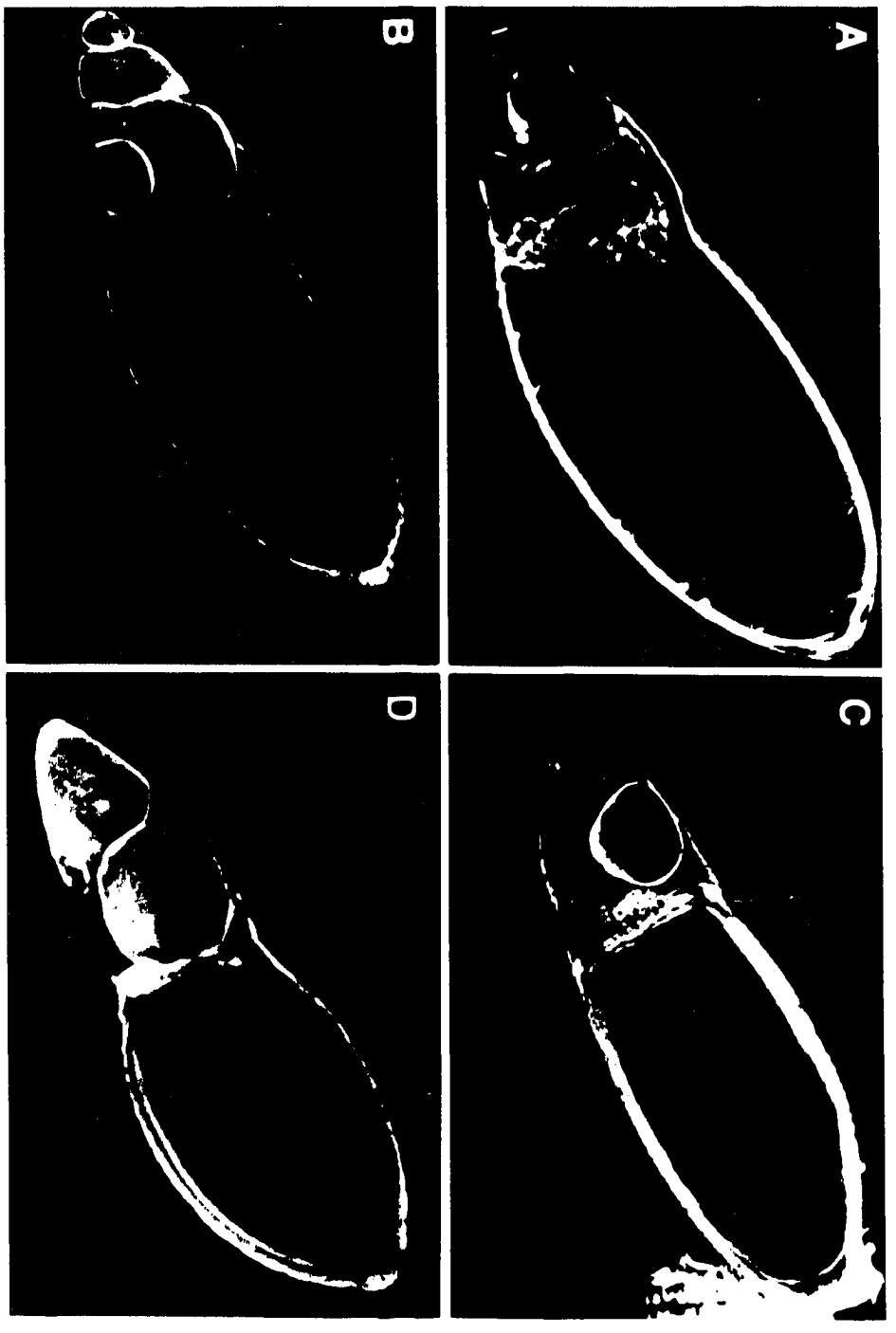
The nurse cell cytoplasm transport defect of sn^{G409E} was enhanced by mutations in *quail* (Table 5). sn^{G409E} is a fertile allele caused by a missense mutation that changes Glycine 409 to Glutamic acid (Table 4). In egg chambers from sn^{G409E} females, the cytoplasmic actin bundles appeared disorganized, the nurse cell cytoplasm was not completely transported and the protein was expressed at nearly wild type levels (Figure 17, Figure 24A). qua^{HM14} is a very weakly fertile allele in which the nurse cell cytoplasmic actin bundles were sparse and mature eggs are about 60-70% the size of wild type eggs (Figure 24C; S. Mahajan-Miklos, 1995). In these *qua* alleles, the quail protein was primarily localized to subcortical actin filaments and was reduced to about 25% of the wild type level (Mahajan-Miklos, 1995). Doubly heterozygous females with one mutant copy of qua^{HM14} and one mutant copy of sn^{G409E} are fertile with no egg chamber phenotype. The weakly fertile *qua* phenotype was not affected by heterozygous sn^{G409E} (data not shown). However, the sn^{G409E} phenotype was enhanced when one mutant copy of qua^{HM14} was crossed into the *sn* background; while sn^{G409E} females were fertile, $sn^{G409E};qua^{HM14}/+$ females were weakly-fertile (Table 5) and produced only about 20% the amount of progeny produced by sn^{G409E} females. sn^{G409E} eggs were about 75% the size and wild type eggs (Figure 24A), whereas $sn^{G409E};qua^{HM14}$ /+ eggs were only 50-70% the size of wild type eggs (Figure 24B, Table 5). The most striking effect was seen in egg chambers from females doubly homozygous for intermediate sn^{G409E} and qua^{HM14} alleles. $sn^{G409E};qua^{HM14}$ females were completely sterile and the eggs were only 50%

Table 5. *sn*^{G409E};*quail*^{HM14} DOUBLE MUTANTS

SINGED	*QUAIL*	FERTILITY
G409E	*WT*	fertile
WT	*HM14*	very weakly fertile
G409E/+	*HM14/+*	fertile
G409E/+	*HM14*	very weakly fertile
G409E	*HM14/+*	semi-fertile
G409E	*HM14*	STERILE

Figure 24. Mutations in *quail* enhance the oogenesis defect in sn^{G409E}

sn^{G409E} females are fertile, however not all of the nurse cell cytoplasm is transported and mature eggs are only about 75% the size of wild type eggs (A). qua^{HM14} is a very weakly allele of quail; nurse cell cytoplasm transport is incomplete and mature eggs are only 60-70% percent the size of wild type eggs (C). The nurse cell cytoplasm transport defect and fertility in sn^{G409E} is sensitive to mutations in *quail*. $sn^{G409E};qua^{HM14}/+$ females are semi-fertile and their eggs were only 50-70% the size of wild type eggs (B). $sn^{G409E};qua^{HM14}$ females were completely sterile and the eggs were only 50% the size of wild type eggs (D).



the size of wild type eggs (Figure 24D, Table 4) Similar enhancement was seen with the intermediate *qua* allele, qua^{WP614} (data not shown).

Overexpression of quail can suppress the *singed* egg phenotype

Nurse cell cytoplasmic actin bundle formation appeared to be sensitive to the level of quail protein expressed. Consequently, I wished to determine whether increasing the level of quail protein expressed in egg chambers could bypass the requirement for fascin function. Transformant lines carrying full length quail transgenes, P[qua$^+$] were generated by Dr. Brenda Knowles. Expression of quail transgenes in the germline was achieved with the pGerm8 expression vector (Serano et al., 1994) which promotes expression beginning at stage 8 of oogenesis. These transformant lines were used to overexpress quail protein in intermediate, strong, and null *sn* mutant backgrounds.

In egg chambers from sn^{G409E} females, nurse cell actin appears disorganized, not all of the nurse cell cytoplasm is transported, and mature eggs are about 75% the size of wild type eggs. However, egg chambers from sn^{G409E} females with three copies of quail (one P-element and two endogenous *quail* genes) appear wild type; the cytoplasmic actin is organized and all of the nurse cell cytoplasm is transported.

To determine whether increasing quail dosage could also rescue the sterility of severe *singed* alleles, I tested for rescue in two sterile *sn* backgrounds, sn^{S289N} and sn^{28}. sn^{S289N} egg chambers express a mutant fascin^{S289N} in nurse cells (Table 4). $sn^{28}$is a null allele that does not express *sn* transcript or protein (Figure 16; Table 4). In egg chambers from both sterile *sn* alleles, the nurse cell cytoplasmic actin bundles are nearly absent, the rapid phase of nurse cell cytoplasm transport is blocked, and mature eggs are only 50% the size of wild type eggs (Figure 17).

sn^{S289N} females with 3 copies of quail were fertile, although the nurse cell cytoplasmic actin bundles appeared somewhat disorganized and mature eggs were only about 75% the size of wild type eggs. sn^{S289N} females with 4 copies of quail were fertile and the cytoplasmic actin bundles appear wild type, nurse cell cytoplasm transport was complete, and mature eggs were the same size as wild type (Figure 25B, D). In a collaboration with Matthew Heinzelman, EM analysis of the nurse cell cytoplasmic actin bundles in these fully rescued sn^{S289N} mutants revealed the same 12 nm periodicity seen on wild type nurse cell actin bundles and on singed-actin bundles formed with purified proteins *in vitro*.

Overexpression of quail was also able to support cytoplasmic actin bundle formation in the absence of fascin. sn^{28} females with three copies of *qua* are semi-fertile and produced about 50% the number of eggs produced by wild type females. Nurse cell actin filament bundles were sparse and disorganized and mature eggs were only about 60-70% the size of wild type eggs. sn^{28} females with four copies of *qua* were fully fertile and the stage 10B nurse cell cytoplasmic actin bundles appeared wild type (Figure 26). However, the nurse cell cytoplasmic actin bundles became disorganized during stages 11-12 of nurse cell cytoplasm transport and mature eggs were only about 80% the size of wild type (Figure 26).

The ability of independent P[qua$^+$] transformant lines to rescue the *sn* oogenesis phenotype was correlated with the level of quail protein produced and the degree of rescue of *qua* mutants (Table 5; Brenda Knowles, personal communication). I examined 8 independent P[qua$^+$] transformant lines for their ability to rescue *sn*. Five independently derived P[qua$^+$] transformant lines that expressed quail protein and rescued *qua* sterility also rescued *sn*

Figure 25. Overexpression of quail can rescue the sterility of sn^{S289N}

Quail transgenes were used to overexpress quail protein in *sn* mutant backgrounds. sn^{S289N} is a sterile allele in which the cytoplasmic actin bundles are absent (A) and mature eggs are only 50% the size of wild type eggs (B). When 4 copies of quail are expressed in the germline cells of sn^{S289N} mutants, nurse cell cytoplasmic actin bundles form (B) and nurse cell cytoplasm transport is complete (D).

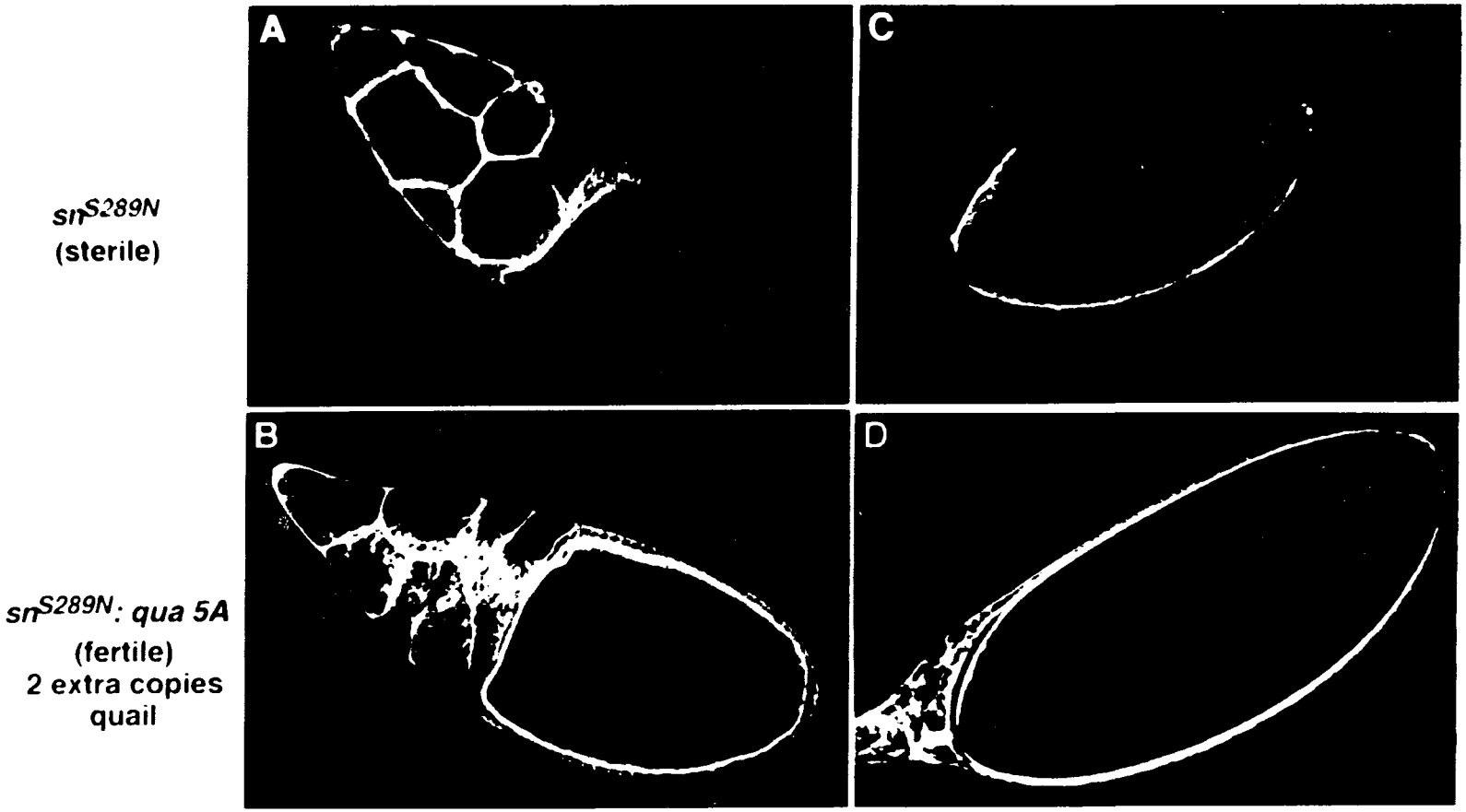


Figure 26. Overexpression of quail can partially rescue the *sn* null, sn^{28}

The null allele, sn^{28}, cytoplasmic actin bundles are absent and eggs are only 50% the size of wild type eggs. However, in the absence of fascin, 4 copies of quail can support the initial cytoplasmic actin bundle formation in stage 10B egg chambers. As nurse cell cytoplasm transport continues in stage 11-12, the cytoplasmic actin bundles appear to fall apart. Although 4 copies of quail can rescue the fertility of sn^{28}, the mature eggs are only about 80% the size of wild type eggs and reflect the defect in the maintenance of cytoplasmic actin bundles throughout nurse cell cytoplasm transport

sterility. In all but one transformant line (25-2A), the degree of rescue correlated with quantity of quail expressed on Western blot analysis. Transformant line 25-2A does not rescue sn^{S289N} or sn^{28}, however, a moderate amount of protein is expressed. Perhaps the timing of quail expression in transformant line 25-2A is disrupted. Since pGerm8 drives germline-specific expression only, quail transformants lines were not tested for their ability to rescue *sn* bristle defects.

Quail headpiece domain is required for *singed* rescue

The cross-linking activity of villin requires two F-actin binding sites. One F-actin binding site is contained in a conserved C-terminal domain called the headpiece. This F-actin binding site is essential for villin's cross-linking activity and has been implicated in the reorganization of the actin cytoskeleton *in vivo* (Friederich et al., 1992). A charged motif, KKEK, in the headpiece F-actin binding domain is required for villin's cross-linking activity. The quail headpiece is also thought to contain a F-actin binding site. In quail's headpiece domain, the corresponding residues are KKQF (Mahajan-Miklos and Cooley, 1994b). Furthermore, bacterially expressed quail protein can cross-link actin filaments. This data suggest that the quail headpiece, like that of villin, contains an F-actin binding site that is required for quail's cross-linking activity.

I examined the ability of quail-headless transformant lines to rescue *sn* oogenesis phenotypes in females with a wild type *qua* background. No rescue was detected in sn^{G409E}; P[$qua^{HL10\text{-}1}$] females that bear two wild type copies of *quail* and carry two copies of headless P[$qua^{HL10\text{-}1}$] transgene. Two copies of

Table 6. Overexpression of quail can rescue *singed* oogenesis defects

		quail^{1374}		*sn*^{G409E} (fertile)		*sn*^{S289N} (sterile)		*sn*^{28} (null, sterile)	
P[quail]	chrom	protein	rescue	1 copy	2 copies	1 copy	2 copies	1 copy	2 copies
2C	2	ND	ND	rescue@	rescue	fertile	fertile	semi fert	fertile
11A	X	++++	++++	rescue	rescue	semi fert*	fertile	semi fert	fertile
1A	3	+	+	no rescue	no rescue	sterile	sterile	sterile	sterile
5A	3	++++	++++	rescue	rescue	semi fert	fertile	semi fert	fertile
44A	3	++++	++++	rescue	rescue	semi fert	fertile	semi fert	fertile
2-4B	3	-/+	-/+	no rescue	50% resc	sterile	sterile	sterile	sterile
2-3A	3	++	++	50% resc$	rescue	sterile	semi fert	sterile	wk fert#
44-3A	3	++	++	50% resc	rescue	semi fert	fertile	sterile	wk fert
25-2A	3	++	+++	50% resc	rescue	sterile	sterile	sterile	sterile
HL10-1	3	++	-	no rescue	no rescue	sterile	sterile	sterile	sterile
HL33	2	++++	-	50% resc	rescue	sterile	sterile	sterile	sterile

@ rescue indicates that eggs achieved wild type size
$ 50% rescue indicates that 50% of the eggs achieved wild type size
* semi fertile indicates that number of progeny obtained was about 50% wild type
# weakly fertile indicates that number or progeny obtained was <25% of wild type

P[qua^{HL10-1}] were not able to rescue the *sn* oogenesis phenotype of sn^{S289N} or sn^{28} (Table 6). In the P[qua^{HL33}] *quail* transformant line, the truncated quail isoform is expressed abundantly from two transgene inserts on the second chromosome (Brenda Knowles, personal communication). sn^{G409E};P[qua^{HL33}]/P[qua^{HL33}] mutants contained homozygous quail loci and 4 copies of the P[qua^{HL33}] transgene. 4 copies of headless P[qua^{HL33}] and two copies of endogenous full length quail can rescue the subtle intermediate egg chamber phenotype of sn^{G409E}, but cannot rescue the sterility of strong *sn* alleles. The headpiece of quail and therefore the crosslinking activity of quail appeared to be required to rescue the sterile *sn* phenotype. The sn^{G409E} mutants have a very subtle phenotype and abundant truncated quail headless protein appeared to be able to increase the organization of cytoplasmic actin bundles and rescued the cytoplasm transport defect.

DISCUSSION

Using genetics manipulation, I was able to alter the dose of singed and quail proteins in nurse cells and demonstrate that cytoplasmic actin bundle formation was sensitive to changes in both of these proteins. Actin bundle formation in the sn^{G409E} mutant is subtly perturbed such that it is sensitive to dosage of quail. Females simultaneously homozygous for both the fertile allele sn^{G409E}, and weakly fertile allele, qua^{HM14}, are sterile and egg chambers from these females do not have nurse cell cytoplasmic actin bundles. Alternatively, overexpression of quail protein in nurse cells can rescue the cytoplasmic actin defect of sn^{G409E}. I further analyzed the ability of quail to rescue more severe, sterile alleles of *sn*. Overexpression of quail can rescue the sterility of sn^{S289N} and sn^{28}. Two extra copies of quail appears to support the assembly and organization of cytoplasmic actin bundles; however,

without fascin, the actin bundles are not as stable as in wild type egg chambers and do not maintain their organization throughout the later stages of nurse cell cytoplasm transport. These genetic analyses suggest that fascin and quail not only act together to stabilize actin filament bundles in nurse cells, but when quail is expressed in excess, the cross-linking activity of fascin appears to be partially redundant with that of quail.

The formation of stable, dense cytoplasmic actin bundles and the completion of nurse cell cytoplasm transport requires some fascin function. Overexpression of quail can rescue sn^{S289N} better than it can rescue a *sn* null, sn^{28} suggesting that fascin^{S289N} retained some actin cross-linking activity. While fascin^{S289N} cannot promote the formation of cytoplasmic actin bundles with wild type levels of quail protein (two copies), the addition of two extra copies of quail appears to allow the fascin^{S289N} to cross-link actin filaments and to leave a 12 nm periodicity seen on EM. In the absence of fascin, overexpression of quail can initiate cytoplasmic actin bundle formation but the bundles are not stable. The late demise of cytoplasmic actin bundles in the null sn^{28} alleles suggests that the nascent quail-actin bundles cannot be stabilized without fascin. The relative stability of cytoplasmic actin bundles in rescued sn^{S289N} suggests that fascin^{S289N} may bind and stabilize the quail-actin bundles. To confirm that the 12 nm periodicity is caused by fascin, I will need to demonstrate that the 12 nm periodicity is absent on actin bundles in quail rescued sn^{28} mutants.

Fascin and quail are not redundant. At wild type protein levels, both are required for nurse cell actin filament bundle formation. Fascin cross-links actin filaments into highly organized, tightly packed, hexagonal arrays (DeRosier and Censullo, 1981). Villin is a calcium regulated protein that cross-links actin filaments into less organized bundles under physiological

calcium concentrations (10^{-7} M) (reviewed in (Mooseker, 1985). The ability of villin to cross-link actin filaments requires a C-terminal headpiece domain. Although quail is not the *Drosophila* homolog of villin, quail is 30% identical to villin, contains a headpiece domain, and appears to contain villin's cross-linking activities. Quail is localized to cytoplasmic actin bundles, is required to form cytoplasmic actin bundles in nurse cells, and bacterially expressed quail can cross link F-actin *in vitro* (Mahajan-Miklos, 1995).

Furthermore, the quail headpiece, and therefore quail's actin cross-linking function, is required to rescue quail and sterile *sn* oogenesis defects. The headpiece of quail is not required to rescue the subtle actin bundle defect in sn^{G409E} if the truncated quail protein is expressed in large excess. Since this truncated quail protein likely does not have actin cross-linking activity, additional quail functions may account for this rescue. The villin headpiece is not required for its ability to cap, sever, or nucleate actin filaments.

Actin bundle assembly and stabilization is a complex process. Actin cross-linking proteins can be important in the initiation, organization, or stability of an actin bundle and therefore, can affect different aspects of actin bundling (Matsudaira et al., 1983). Since actin bundling proteins typically have distinct biochemical properties *in vitro*, then it often has been suggested that the different properties of the two bundling proteins present in a cell are necessary in the *de novo* formation of actin bundles. Both villin and fimbrin are implicated in the formation of actin bundles in intestinal microvilli. Quail has homology to villin and fascin and fimbrin are both known to cross-link actin filaments into highly ordered, hexagonal arrays. In microvillar model systems it has been suggested that villin initiates actin bundle formation and that fimbrin stabilizes the actin bundles. The ability of quail to stimulate cytoplasmic actin bundle formation in the absence of fascin is

similar to villin's ability to initiate the formation of microvillar-like structures in fibroblasts. It is tempting to speculate that in nurse cells, quail may initiate cytoplasmic actin bundle formation and fascin may organize or stabilize the quail-actin bundles. Although the different properties of fascin and quail appear to support optimal actin bundle formation, quail is sufficient to produce cytoplasmic actin bundles in nurse cells that can support fertile egg development.

While the full implications of this result are difficult to appreciate at this time, I have generated an excellent *in vivo* system to study the difference between actin bundles made with a single bundling protein and those made with two bundling proteins. EM analysis of cytoplasmic actin bundles in quail-rescued *singed* egg chambers is currently being examined in collaboration with Matthew Heinzelman. It will also be interesting to look at actin bundle formation *in vitro* with mixtures containing different ratios of purified *Drosophila* fascin and purified quail.

P[singed] transformants are not available to test whether overexpression of fascin can rescue *qua* mutants. However, fascin is already expressed in excess in nurse cells. In weak *sn* alleles, wild type fascin is reduced to about 25% of wild type levels without having any visible effect on cytoplasmic actin bundles formation and cytoplasm transport (Cant et al., 1994). Furthermore, I do not know if quail contains any of villin's ability to cap, sever, or nucleate actin filaments. If quail uses any of these activities to initiate actin filament bundle assembly, then fascin will not be able to compensate for these functions.

CONCLUSIONS AND FUTURE DIRECTIONS

Both genetic and biochemical data presented in this dissertation provide strong evidence that *Drosophila* singed is a homolog of the actin bundling protein fascin. *Singed* is required for actin bundle formation in developing bristles and during oogenesis, and bacterially expressed singed protein bundles actin filaments *in vitro* with the same 12 nm periodicity and stoichiometry as described for fascin.

Using *in vivo* mutagenesis, I have generated three new *singed* alleles with mutations in fascin and have identified the amino acid(s) affected in each allele. The regions affected in these mutants can now be targeted for more detailed biochemical characterization of fascin. Since the sn^{S289N} suppressor mutation S251F appears to restore fascin function *in vivo*, it will be interesting to determine if the S251F mutation can restore solubility to $fascin^{S289N}$ *in vitro*.

The new *singed* alleles were useful in demonstrating that fascin acts together with quail protein to promote cytoplasmic actin bundle formation in nurse cells. Although the different properties of fascin and quail appear to support optimal actin bundle formation, quail is sufficient to produce cytoplasmic actin bundles in nurse cells. Quail expression was restricted to the germline tissue. By expressing quail in developing bristles I can analyze whether quail is sufficient for actin bundle formation in bristle development also. The reciprocal experiment with overexpressed fascin will require germ-line P[singed] transformants.

P-element mediated germline transformation with singed will be critical to future analysis of fascin. *singed* transformants with the endogenous promoter would provide a model system for studying the role of

fascin in both bristle development and oogensis. However, the endogenous *sn* promoter is not characterized and it is not known how much genomic DNA would be needed in the transformation vector. To study oogenesis, *singed* expression can be driven by the pGerm8 expression vector (Serano et al., 1994). These P[singed] transgenes, expressed from stage 8 onward, should allow rescue of the *singed* cytoplasm transport phenotypes and determine whether both the cytoplasm transport and micropyle morphology defects in *sn* mutants are rescued by germline expression of fascin as opposed to somatic follicle cell expression. P[singed] transgenes will allow overexpression of fascin in the germline in order to determine if an overexpression phenotype is observed and if *quail* phenotypes can be rescued by overexpressing fascin. Transformation can also be used to express mutant fascins. Now that I have determined three amino acids critical to fascin function, site-directed mutagenesis and germline transformation can be used to analyze these and additional mutant fascins *in vivo*. Finally, it will be interesting to express truncated fascin proteins. Truncated fascin proteins that contain only a single actin binding site may have a dominant negative effect.

Both nurse cell cytoplasm transport and bristle extension provide excellent *in vivo* models for the functional analysis of actin binding proteins that complement in vitro biochemical characterization. Future analysis will be directed at trying to determine the actin binding domains of fascin and understanding the complex interactions between actin and its regulating proteins. Fascins exist in a variety of organisms and a variety of tissues. Understanding the role of fascin in *Drosophila* will likely provide insight into complex cytoskeletal dynamics in a number of different systems.

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